# NEUROMELANIN AND ALPHA-SYNUCLEIN

# MODULATION OF INFLAMMATORY SIGNALING

# IN HUMAN ASTROGLIAL CELLS

By

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and bad. I would like to dedicate my work to my little sister, Nika, whom I love with all my heart and can't wait to see her as a young, successful lady one day.

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# CHAPTER I

## INTRODUCTION

Parkinson's disease (PD) is a chronic, degenerative neurological disorder that affects one in 100 people over age 60 (Lang and Lozano, 1998). PD is progressive with a mean age at onset of 55, with an incidence that increases with age (Dauer and Przedborski, 2003). There is still no objective test, or biomarker, for Parkinson's, so the rate of misdiagnosis can be relatively high. Recent research indicates that at least one million people in the United States, and more than five million worldwide, have Parkinson's disease (Fahn, 2003).

Parkinson's disease was first characterized by an English doctor, James Parkinson, in 1817 (Aronson 1986). Today, we understand Parkinson to be a disorder of the central nervous system (CNS) that results from the loss of neuronal cells predominantly in the substantia nigra (SN) pars compacta (SNpc). Because the SNpc cells produce dopamine, a chemical messenger responsible for coordination of movement, a loss of these neurons causes a fall in dopamine levels in the striatum, ultimately leaving patients less able to control their movement. This leads to the “cardinal” symptoms of PD, such as resting tremor, bradykinesia, postural instability, and rigidity (Savitt et al., 2006). Some of the

"non-motor" symptoms include cognitive impairment, ranging from mild memory difficulties to dementia, and mood disorders, such as depression and anxiety (Hoehn and Yahr, 1998). Also common are sleep difficulties, loss of sense of smell, speech and swallowing problems, unexplained pains, drooling, and constipation (Savica et al., 2010).

The exact mechanism responsible for the neuronal loss in PD remains unclear; however, neuroinflammation appears to be involved (Hirsch et al., 1998; Hirsch et al., 2003; Hunot and Hirsch, 2003; McGeer et al., 2001; Whitton, 2007; Wyss-Coray and Mucke, 2002). Neuroinflammation seems to be instrumental given the increase in reactive glia and increased expression of inflammatory molecules in astroglia and microglia (Forno et al., 1992; Miklossy et al., 2006). Of these inflammatory molecules, the pro-inflammatory cytokines tumor necrosis factor – alpha (TNFα) and interleukin-1beta (IL-1β) are elevated in the striatum and cerebrospinal fluid (CSF) of PD patients. Additionally, TNFα and IL-1β mediate cell death in a population of dopaminergic neurons and cause PD motor disabilities, possibly indicating an involvement of these cytokines in the pathology of the disease (De Lella Ezcurra et al., 2010; McGuire et al., 2001).

In terms of cell types contributing to PD neuropathogenesis, microglia are shown to be involved (Cicchetti et al., 2002; Ghosh et al., 2007; McGeer and McGeer, 1998b). For instance, analysis of PD patients by utilizing specific makers of activated microglia shows clear evidence of microglial activation in the SN (Cicchetti et al., 2002; Gerhard et al., 2006). Large numbers of human leukocyte antigen-DR (HLA-DR) and CD11b-positive microglia are found in the SN in the brains of these patients (McGeer et al., 1988). Although past research has not thoroughly investigated the role of astrocytes in PD, increasing evidence points to astrocytes as contributors to dopaminergic neuronal degeneration (Henning et al.,

2008; McGeer and McGeer, 2008; Yasuda et al., 2008). Hence, a fuller characterization of astrocytes under PD-related conditions will enhance our understanding of PD-associated neuroinflammation. This dissertation is an investigation of two PD-associated molecules, neuromelanin (NM) and α-synuclein, and their effect on human astroglial cells. Reported first are the effects of NM and α-synuclein on neuroinflammatory molecules including chemokines (CXCL10) and inducible nitric oxide synthase (iNOS) in human astroglial cells. Given these inflammatory molecules are dependent upon the transcription factor, nuclear factor κB (NF-κB), the effects of NM and α-synuclein on NF-κB are also investigated. The purpose of this study is to understand the actions of NM and α-synuclein on the modulation of pro-inflammatory signaling pathways in astroglial cells. The rationale for examining the roles of NM and α-synuclein is to provide a foundation from which future research can investigate inflammatory-related therapeutic drugs for the treatment and prevention of PD-associated neuroinflammation.

# CHAPTER II

# REVIEW OF LITERATURE

## 2.1 Neuropathogenesis of Parkinson's disease (PD)

Parkinson's disease affects the pigmented nigrostriatal dopaminergic neurons and results in a progressive neurodegeneration of these neurons. PD is distinguished as the second most common neurodegenerative disorder after Alzheimer's disease with a prevalence of 0.1% of the global population (Whitton, 2007). The symptoms of PD typically appear when loss of at least 50% of the dopaminergic neurons in the SNpc occurs, leading to an over 80% reduction in dopamine levels in the striatum (Deumens et al., 2002; Lang and Lozano, 1998). Depleted levels of dopamine, a neurotransmitter that dampens muscle jerkiness and exerts smooth muscle movement, causes unbalanced levels between dopamine and acetylcholine in the basal ganglia (Figure 1). The imbalance of neurotransmitters ultimately prevents the basal ganglia from exerting controlled muscle contractions. Uncontrolled movement will eventually lead to high muscle tension, and incidents of tremor and slow movement. The clinical manifestations of PD include akinesia, bradykinesia, a rhythmic involuntary tremor at rest ('pill rolling movement'), postural instability, and extrapyramidal rigidity in which major muscle groups become stiff.

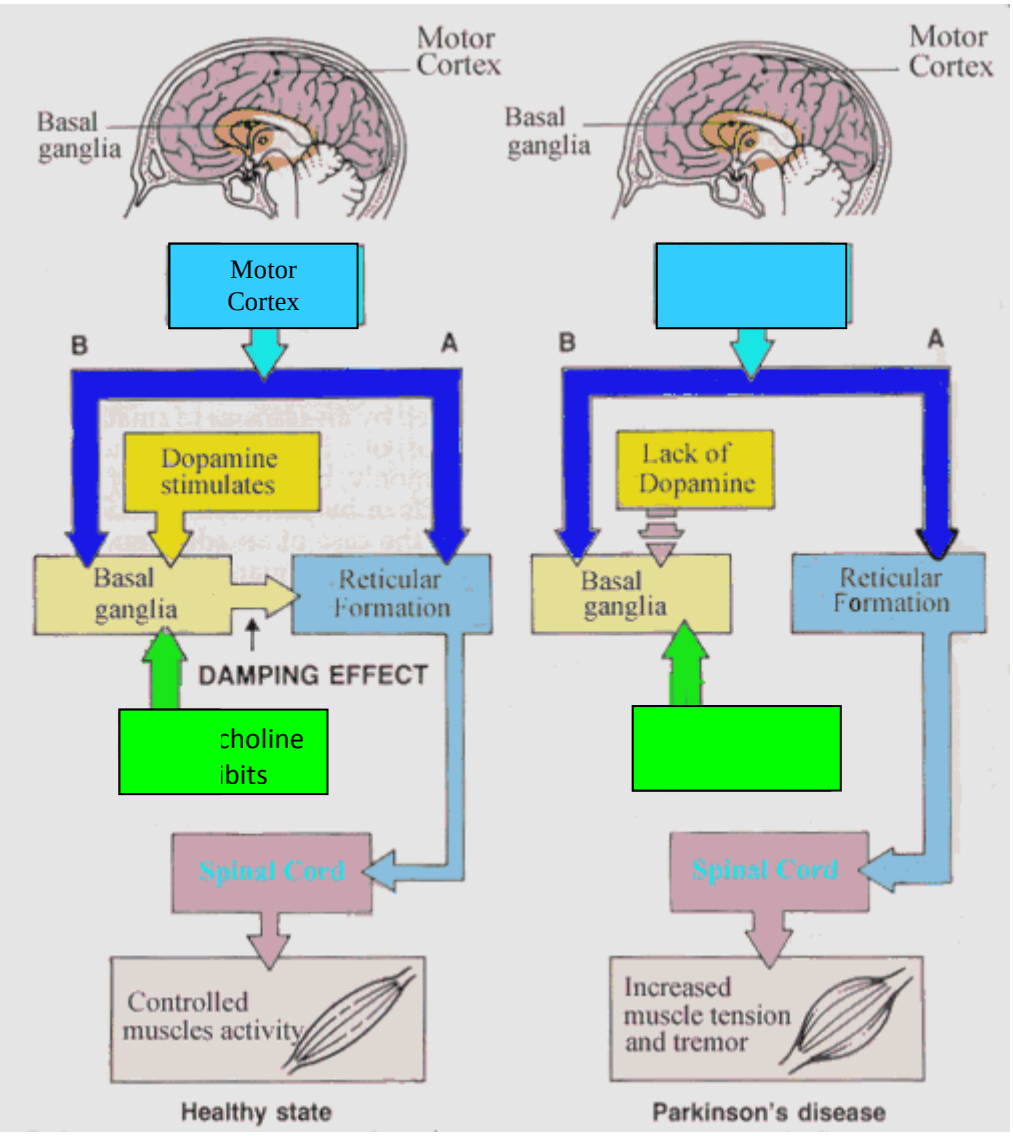


**Figure 1. Pathway scheme of healthy state of brain versus Parkinson's disease brain.** During movement, signals pass from the brain's cortex, via reticular formation and spinal cord (pathway **A),** to muscles, which contract. Other signals pass, by pathway B, to the basal ganglia; which dampen the signals in pathway A, reducing muscle tone so that movement is not jerky. Dopamine is needed for this damping effect. Another transmitter, acetylcholine, inhibits the damping effect. A lack of dopamine will reduce or abolish the damping effect, eventually resulting in an increase in muscle tension and tremor.
**http://www.holisticonline.com/remedies/parkinson/pd_brain.htm**

### 2.1.1 Neuroinflammation in PD

While the exact mechanism by which PD causes neuropathology is not fully understood, new information has emerged in the past 15 years to strongly suggest that inflammation-derived oxidative stress (Hastings et al., 1996; Mizuno et al., 2008; Onyango, 2008), mitochondrial dysfunction (Mizuno et al., 2008; Onyango, 2008) and cytokine-dependent toxicity (Hirsch et al., 1998; Hirsch et al., 2003; Hunot and Hirsch, 2003; McGeer et al., 2001; Whitton, 2007; Wyss-Coray and Mucke, 2002) may contribute to the neuronal degeneration and the progression of PD. Numerous studies have implicated neuroinflammation in the pathogenesis of PD. For example, men with high plasma concentrations of IL-6 have increased risk for PD (Chen et al., 2008). Several genetic studies have analyzed the relationship between polymorphism in neuroinflammation-associated genes and PD. For instance, Wahner and colleagues (2007) found that the risk of PD is increased two-fold in patients with the homozygous variant of IL-1β at position -511 and TNFα at position -308 and that this risk is increased three-fold in carriers for both variants (Wahner et al., 2007). Perhaps the most convincing evidence to support the claim that inflammation contributes to PD comes from epidemiological studies (Chen et al., 2003; McGeer and McGeer, 1998b). A large study indicated that the incidence of PD in chronic users of non-aspirin non-steriodal anti-inflammatory drugs (NSAIDS), which scavenge free oxygen radicals and inhibit cyclo-oxygenase activity, was about 50% lower than that of age-matched non-users (Chen et al., 2003). This suggests that inflammation may be a key player in PD.

Neuroinflammation may also be evident in PD from studies of cellular and molecular examinations. For instance, an increase in reactive glia and increase in

expression of inflammatory molecules in astroglia and microglia has been observed in the SN and striatum in PD patients (Forno et al., 1992; Miklossy et al., 2006) (Figure 2).

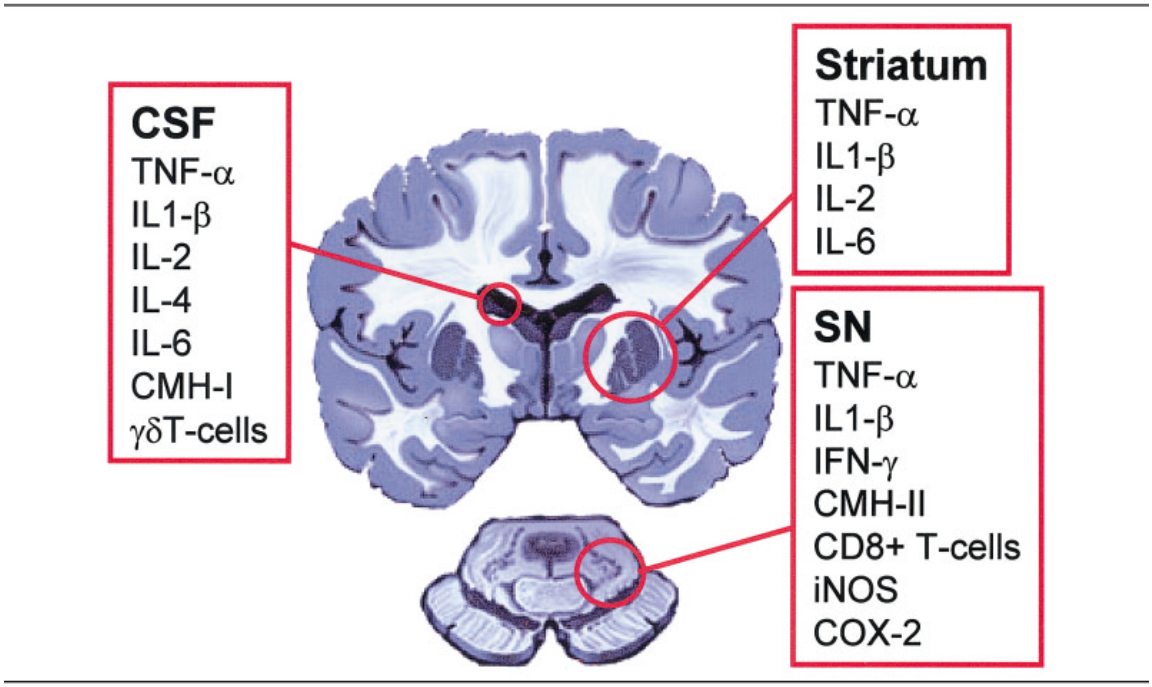


**Figure 2: Inflammatory-related changes in Parkinson's disease brains**. Cytokines found to be upregulated in the outlined regions (cerebral spinal fluid, substantia nigra, striatum) of the PD brain compared to control subjects. Image adapted from *Annals of Neurology* with permission (Hunot and Hirsch, 2003)

Parkinson's disease is primarily a neurodegenerative disease; therefore, past research has thoroughly investigated the involvement of dopaminergic neurons as well as microglial cells. To date, little research has been done to investigate the role of another type of CNS cell: the astrocytes.

### 2.1.2 Role of astrocytes in normal vs PD brain

Astrocytes are the major glial component of the CNS and constitute up to 50% of brain volume (Tower and Young, 1973). Under physiological states, astrocytes support neurons, both physically as cellular matrix and physiologically by providing a stable

environment and growth factors (Anderson et al., 2003; Chen et al., 2006). Astrocytes ensure neuronal homeostasis by taking up excess neurotransmitters and buffering the ionic content of the extracellular environment in the brain (Fields and Stevens-Graham, 2002). Additionally, astrocytes are known to play an important role in antioxidant defense in the brain (Morale et al., 2006).

Astrocytic reactions also represent an important feature under pathophysiological conditions. In addition to microglia, astrocytes have been shown to be involved in brain inflammation (Carpentier et al., 2005) in various neurodegenerative and neurological disorders (Tanuma et al., 2006; van Marle et al., 2004; Xia et al., 2000). However, the role of astrocytes in the development of PD is still unclear. In PD studies, astroglial activation, as measured by glial fibrillary acidic protein (GFAP) expression, has been reported in the SN and striatum of rodents exposed to drug-induced parkinsonism (Rodrigues et al., 2001). Furthermore, a 30% increase in the density of astroglial cells in the SN of PD patients at post-mortem was detected by glutathione peroxidase-positive cell analysis (Damier et al., 1993). Diseased brains of PD patients and cultured cells also illustrated astrocyte participation in the inflammatory response (Figure 3) by their production of noxious factors such as proinflammatory cytokines which damage CNS cells. Specifically, inflammatory-changes in cytokine IL-1α/β, IL-6, IFNγ, TNFα, and chemokines monocyte chemotactic protein -1 (MCP-1), macrophage inflammatory protein -1α (MIP-1α), MIP-1β expression have been reported in PD patients as well as in PD animal models (Hirsch et al., 2003; Hunot and Hirsch, 2003; Kalkonde et al., 2007; Knott et al., 2000; McGeer and McGeer, 2008; Yasuda et al., 2008). Therefore, astroglial cells are implicated in PD neuropathology, but further research is needed. Given the

roles of astrocytes in the CNS, an impairment of astrocyte-neuron crosstalk may contribute to disease progression and impair the recovery process.

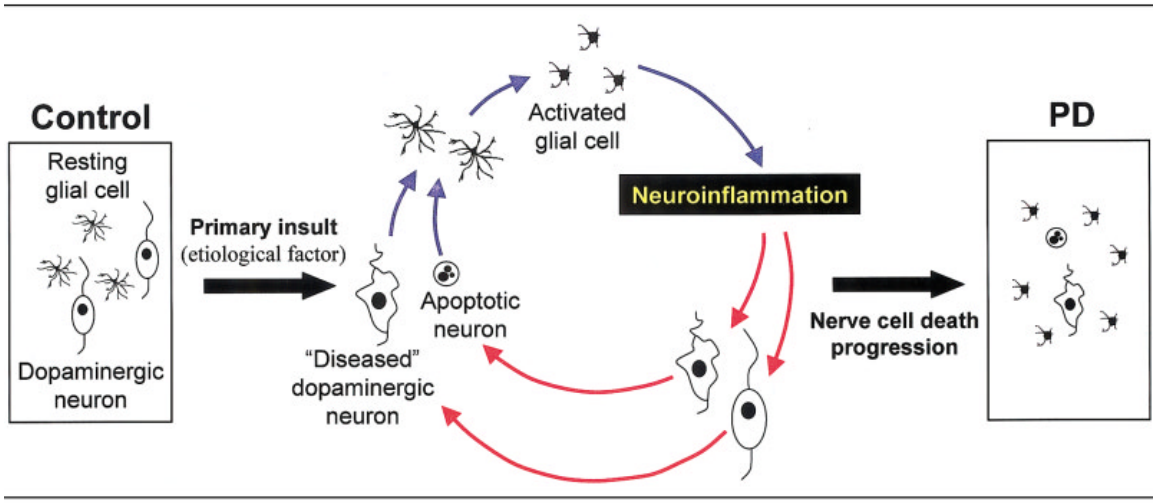


**Figure 3: Representation of the hypothesized role of glial cells and inflammation in nerve cell death progression in Parkinson's disease (PD)**. In the brain of control subjects (left), resting glial cells surround healthy dopaminergic neurons. After an initiating insult (the causative factors), some dopaminergic neurons degenerate by apoptosis and others are damaged or stressed ("diseased" dopaminergic neurons). Through an unknown mechanism, these injured neurons trigger activation of glial cells, which is illustrated by morphological changes (blue arrows). Reactive glial cells secrete proinflammatory cytokines and inflammatory and/or immune-associated markers (blue arrows). In turn, this neuroinflammation can damage more dopaminergic neurons (red arrows), which will further activate the glial cells. This vicious circle then could contribute to the propagation of nerve cell death and the development of parkinsonism (right). Image copied from *Annals of Neurology* with permission (Hunot and Hirsch, 2003).

## 2.2 The Tumor Necrosis Factor (TNF) family

### 2.2.1 TNF cytokine family

The Tumor Necrosis Factor (TNF) family is composed of 19 related cytokines that play a pivotal role in orchestrating innate inflammatory responses (Pfeffer, 2003). Following its isolation in 1975 and the cloning of its gene in 1984, studies showed two different TNFs in the family, TNFα and lymphotoxin (now known as TNFβ) (Aggarwal et al., 1984; Aggarwal et al., 1985b). In 1985, a 30% amino acid similarity between the

two factors was found (Aggarwal et al., 1985b). Additionally, the binding of TNFα to its receptor and its displacement by TNFβ confirmed the functional homology between the two proteins (Aggarwal et al., 1985a). TNFβ is produced by lymphocytes, but in the CNS, TNFα is produced by astrocytes, microglia, and neuronal cells. As summarized in Ernandez and Mayadas (2009), TNFα is synthesized as a propeptide with a long and atypical signal sequence, which is absent from the mature secreted cytokine. A short hydrophobic stretch of amino acids serves to anchor the propeptide in lipid bilayers. This 26 kDa membrane-integrated protein (mTNFα) is released from the cell via proteolytic cleavage by TACE, the metalloprotease TNF-Alpha Converting Enzyme. The soluble 17 kDa protein (sTNFα) is secreted after cleavage of the propeptide. In both the cell-associated and secreted forms of TNFα, trimerization is required for biological activity. Both these forms are biologically active, and the cell-associated form is thought to be responsible for cell-to-cell contact (Kriegler et al., 1988). The primary role of TNFα is in the regulation of immune cells. Tumor necrosis factor α is able to induce apoptotic cell death and inflammation, and inhibit tumorigenesis and viral replication (Balkwill, 2009; Ernandez and Mayadas, 2009).

### 2.2.2 TNFα receptors and CNS expression

TNFα signals through two distinct cell-surface receptors: TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) (Palladino et al., 2003). Characterization of TNFR's has been summarized by Palladino et al. (2003) as follows: TNFR1 is the primary receptor for the soluble form of TNFα, whereas TNFR2 is the primary receptor for membrane-bound TNFα. Similar to TNFα, both receptors have to form homotrimers to be biologically active. Both receptors are transmembrane glycoproteins with multiple

cysteine-rich repeats in the extracellular N-terminal domains. Although their extracellular domains share structural and functional homology, their intracellular domains are distinct, and transduce their signals through both overlapping and distinct pathways (Figure 4). The main intracellular structural difference between TNFR1 and TNFR2 is the presence of the death domain (DD) in TNFR1, which is not present in TNFR2 (Palladino et al., 2003). When TNFR1 is not activated, the DD is occupied by the SODD (silencer of DD protein), which prevents activation of the receptor in the absence of the ligand. When TNFα binds to TNFR1, this induces the release of SODD and allows the TRADD (TNFR associated DD) to interact with the DD. TRADD is an essential partner of TNFR1 for signal transduction. TRADD can recruit the downstream adapter molecule FADD (fas-associated DD), which initiates the caspase pathway responsible for apoptotic cell death. It can also interact directly with the TNF receptor-associated factor 2 protein (TRAF2). TRAF2 can then interact with RIP (receptor-interacting protein) to mediate lymphocyte apoptosis. TRAF2 can also trigger the activation of several inflammatory signaling pathways, including NF-κB, AP-1, c-Jun N-terminal kinase stress kinases, and p38 mitogen-activated protein kinase (MAPK), which control TNF-induced gene expression (Ernandez and Mayadas, 2009; Palladino et al., 2003). These inflammatory pathways are primarily activated by TNFR1. Some of the proinflammatory properties of TNFα include induction of chemokines (monocyte-chemoattractant protein-1, CXCL10 or IP10), cytokines (IL-1, IL-6), and other inflammatory mediators (reactive oxygen species, nitric oxide) (Tansey et al., 2007). Some reports indicate that TNFR1 mediates apoptosis and TNFR2 mediates proliferation;

thers suggest that the two TNFRs transduce their signals cooperatively (Mukhopadhyay et al., 2001; Weiss et al., 1998).

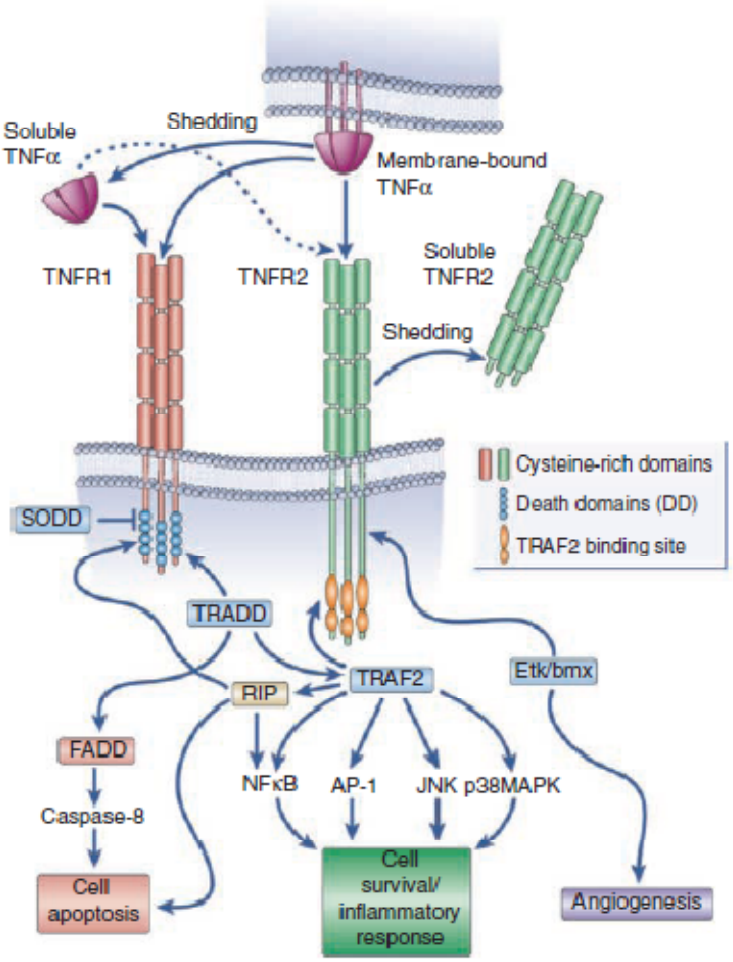


**Figure 4**. **TNFα receptors and receptor-signaling pathways.** Diagram illustrating TNFR1 and TNFR2 and their role in signaling pathways. The binding of membrane-bound or soluble TNFα induces the activation of TNFR2 and the release of SODD from TNFR1 to allow TRADD to interact with DD. Downstream proteins and enzymes interact to allow signaling of TNFα receptors. Activation of the TNFRs ultimately lead to cell apoptosis or inflammatory responses. SODD, silencer of death domain protein; TRADD, TNF receptor-associated death domain protein; TRAF2, TNF receptor-associated factor 2 protein; Etk/bmx, endothelial/epithelial tyrosine kinase; FADD, Fas-associated death domain protein; RIP, receptor interacting protein; JNK, c-Jun N-terminal kinase. Image copied from *Kidney International* with permission (Ernandez and Mayadas, 2009).

All nucleated cells express TNF receptors, although their distribution varies with cell type (Palladino et al., 2003). TNFR1 is expressed constitutively on most cell types,

whereas expression of TNFR2 is highly regulated (Boka et al., 1994). In the CNS, for example, TNFR1 is expressed on nigrostriatal dopaminergic neurons (Boka et al., 1994; McGuire et al., 2001; Mogi et al., 2000) as well as on astroglial cells (Fernandes et al., 2006; Mennini et al., 2004; Sairanen et al., 2001; Yin et al., 2004). TNFR2 is expressed primarily by immune cells including microglia (Dopp et al., 1997), but also reported in numerous other cell types including dopaminergic neurons (McGuire et al., 2001).

### 2.2.3 Overview of TNFα's role in PD

TNFα is expressed at a minimal level in the CNS under basal conditions but becomes elevated after an insult, infection, or injury (Breder et al., 1993; Tansey et al., 2007). Dysregulation of TNFα production has been implicated in a variety of human diseases, such as Alzheimer's disease (Fillit et al., 1991), multiple sclerosis (Hofman et al., 1989), human immunodeficiency virus (Grimaldi et al., 1991) as well as cancer (Bersani et al., 1986). In PD, in particular, elevated TNFα and the contribution to dopaminergic neuronal dysfunction and cell death has been observed. For example, TNFα is upregulated in the striatum and SN of post-mortem brains of PD patients (Boka et al., 1994; Mogi et al., 1994b) and increased in the serum and cerebrospinal fluid of PD patients compared to healthy individuals (Dobbs et al., 1999; Mogi et al., 1994b). Also, in *in vivo* PD models TNFα is up-regulated in the striatum and SN (Mogi et al., 1999). Additionally, significantly elevated mRNA and protein levels of TNFα have been detected in the rodent midbrain SN within hours of *in vivo* administration of parkinsonism-inducing neurotoxins (Ferger et al., 2004; Mogi et al., 1999; Rousselet et al., 2002; Sriram et al., 2002). Moreover, plasma TNFα levels remain elevated one year post administration of neurotoxin in non-human primates, showing its possible

involvement or influence in chronic inflammation (Barcia et al., 2005). Importantly, TNFα was highly toxic to dopaminergic neurons in both *in vitro* (De Lella Ezcurra et al., 2010; McGuire et al., 2001) and *in vivo* (Carvey et al., 2005) studies. Furthermore, results from genetic evidence reveal that a single nucleotide polymorphism in the TNFα promoter is over-expressed, resulting in higher than normal TNFα production in a group of early onset PD patients (Nishimura et al., 2001). Studies involving TNFα knockout and inhibitor proteins also support the role of TNFα in PD. For example, MPTP-induced neurotoxicity was mitigated in mice lacking TNFα or both TNF receptors (Ferger et al., 2004; Sriram et al., 2002). Chronic infusion of TNFα inhibitor proteins into rat SNpc displayed significant dopaminergic neuroprotective properties from parkinsonism-inducing neurotoxin (McCoy et al., 2006). Furthermore, glial cells in the SN of PD patients have enhanced TNFα production (Boka et al., 1994). In principle, two mechanisms could account for TNFα's neurotoxicity: either a direct mechanism through receptor binding on dopaminergic neuron or an indirect mechanism through glial-cell activation and expression of inflammatory factors. Together, these studies utilizing histopathologic, epidemiologic, and pharmacologic analysis support the concept that TNFα is a critical mediator of dopamergic neurodegeneration in PD.

## 2.3 The Interleukin (IL) -1 family

### 2.3.1 IL-1 cytokine family

The Interleukin (IL)-1 cytokine family is compromised of at least 11 ligands which play an important role in inflammation and host defense (Basu et al., 2004). IL-1 was first described in 1972 (Gery et al., 1972) and cloned in humans in 1985 (March et

al., 1985). The cloning of IL-1 showed two separate genes encoding two different types of IL-1, IL-1α and IL-1β (Subramaniam et al., 2004). These agonists display amino acid homology of 26% in humans (Dower et al., 1986). Interleukin-1α and IL-1β are synthesized as large precursor proteins (31 kDa) by many cell types in the peripheral and central immune system, including monocytes, macrophages, neutrophils, hepatocytes, microglia, and astrocytes (Basu et al., 2004; Simi et al., 2007). Pro-IL-1α is biologically active and cleaved by calpain to generate the mature 23 kDa protein. Both forms of IL-1α are biologically active and remain mostly intracellular, unless released following cell death (Allan et al., 2005). In contrast, pro-IL-1β is biologically inactive and requires caspase-1 (also known as ICE, IL-1β converting enzyme) for cleavage into an active 17 kDa protein, which is secreted out of the cell (Allan et al., 2005).

The IL-1 family also has a third member that has been well characterized as a naturally occurring competitive IL-1 receptor antagonist, IL-1RA (Hannum et al., 1990). IL-1RA is produced by the same cells as IL-1 and is expressed as an intracellular isoform or a secreted isoform. The secreted isoform of IL-1RA (22 kDa) is the only form reported in the brain (Hannum et al., 1990). The receptor antagonist binds to the IL-1 receptor with the same affinity as IL-1 but does not trigger signal transduction. Therefore, IL-1RA's main function is to regulate the effects of IL-1 by blocking receptors. Administration of IL-1RA in experimental animals resulted in neuroprotection following a number of insults including ischemia and excitotoxicity (Hagan et al., 1996; Yang et al., 1997).

The biological activity of IL-1 is tightly controlled under physiolocial conditions. Upon stimulation, IL-1 is produced by a large variety of cells and acts in a

paracrine/autocrine fashion on target cells. Roles of IL-1 include the regulation of metabolism, cytokine proteins, vascular system, and hematological molecules, with the primary role of IL-1 being a mediator of innate immunity with numerous CNS biological actions, including inflammation (Dinarello, 1988; Dinarello and Wolff, 1993; Dinarello, 1996; Hallegua and Weisman, 2002).

### 2.3.2 IL-1 Receptors and CNS expression

The IL-1 family contains two forms of receptors (yielding ten receptor subtypes) that are from two different genes but display similarities in their transmembrane and extracellular domains (Martin and Falk, 1997). The effects of IL-1α and IL-1β are mediated though actions on the type I IL-1 receptor, IL-1R1 (80 kDa), which exists in both membrane-bound and soluble forms (Subramaniam et al., 2004). There is also a type II IL-1 receptor, IL-1R2 (60-65 kDa), but this receptor lacks an intracellular signaling domain so no downstream signaling is initiated when IL-1 binds. Therefore, IL-1R2 is believed to act as a "decoy" receptor by preventing access of IL-1 to the functional IL-1R1 (Subramaniam et al., 2004).

Signal transduction requires the binding of IL-1β to membrane-bound IL-1R1, which then associates with the membrane-bound IL-1 receptor accessory protein (IL-1RAcP) (Rothwell and Luheshi, 2000). The complex then recruits a number of intracellular adaptor molecules, including myeloid differentiation factor 88 (MyD88), IL-1R-associated kinases (IRAK), and TNFR-associated factor 6 (TRAF6) to activate signaling via NF-κB, p38, p42/44, ERK1/2, and JNK MAPK (Figure 5), which will induce inflammatory-associated genes encoding chemokines (CCL2), growth factors

(nerve growth factor), adhesion molecules (matrix metalloproteinases) and cytokines (IL-6, TNF) (Basu et al., 2004; John et al., 2005; Allan et al., 2005).

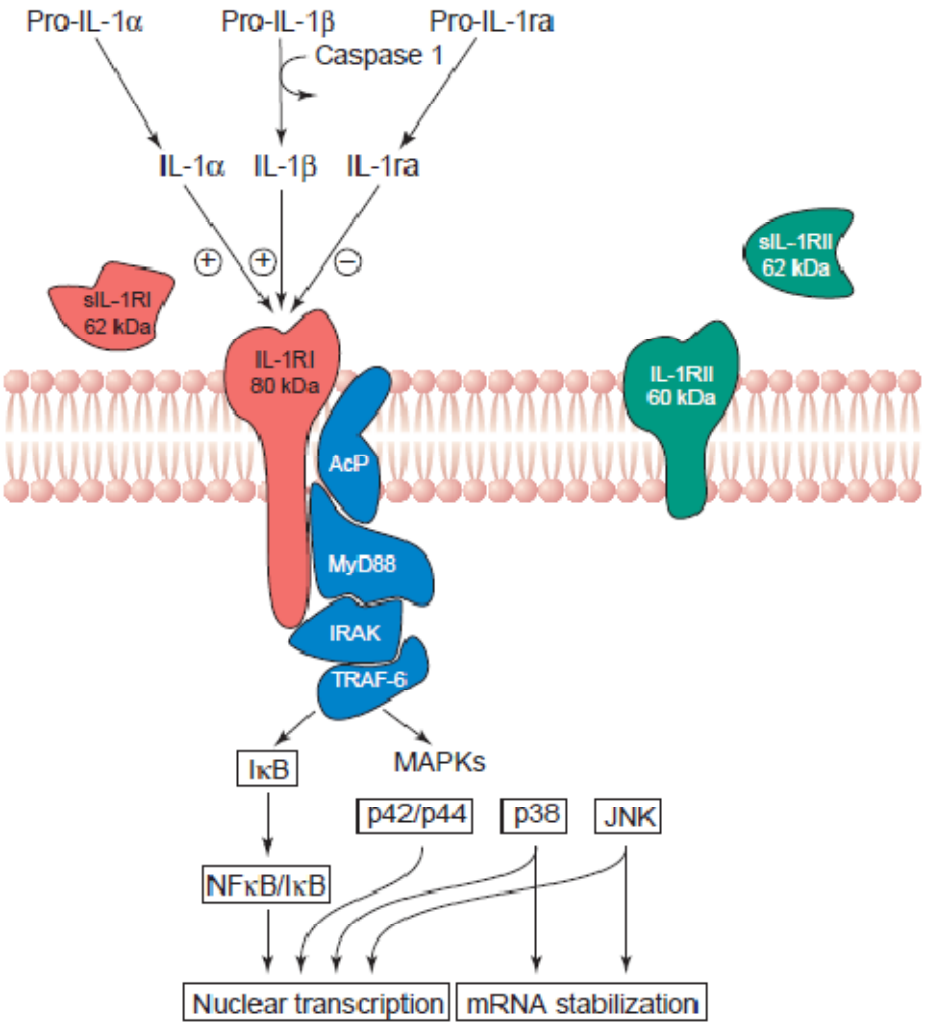


**Figure 5. Actions of IL-1.** The IL-1 cytokine family members include two ligands (IL-1α and IL-1β), an antagonist (IL-1RA), and two receptors (IL-1R type 1 and IL-1R type 2). The binding of IL-1α/β/ra to IL-1RI allows proteins and kinase to interact with the receptor and induce downstream signaling. IL-1RII also binds IL-1, but lacks an intracellular domain and does not initiate signal transduction. AcP, accessory protein; MAP, mitogen-activated protein; IRAK, interleukin-1 receptor associated kinase; TRAF, tumour necrosis factor receptor associated factor; NF-kB, nuclear factor-kB; JNK, c-jun amino terminal kinase .Image copied from *Trends in Neuroscience* with permission (Rothwell and Luheshi, 2000)

All members of the IL-1 family (IL-1α, IL-1β, IL-1R1, IL-1R2, and IL-1RA) have been observed in the CNS (Rothwell and Luheshi, 2000). Interleukin -1α and IL-1β

are expressed at low levels in the healthy CNS, but are upregulated within minutes at the mRNA level and within hours at the protein level in response to neurotoxic stimuli (Allan et al., 2005; Simi et al., 2007). Cellular sources of IL-1α and IL-1β and receptors IL-1R1 and IL-R2 in the CNS appear to predominately be microglia and astrocytes (Davies et al., 1999; Pearson et al., 1999) as well as neurons and oligodendroglia (Blasi et al., 1999).

### 2.3.3 Overview of IL-1β in PD

Interleukin-1β in particular has been implicated in a number of human diseases, including multiple sclerosis (McFarland and Martin, 2007), myasthenia gravis (Huang et al., 1998), epilepsy (Sheng et al., 1994), HIV-associated dementia (Zhao et al., 2001), Parkinson's disease (Mogi et al., 1994a), and Alzheimer's disease (Nicoll et al., 2000). A significant increase in IL-1β within the striatum and SN of post-mortem patients as been observed (Hunot et al., 1999; Mogi et al., 1994a). Similary, IL-1β is elevated in the CSF of PD patients compared to healthy patients (Blum-Degen et al., 1995). Cell culture and animal studies also support an involvement of this pro-inflammatory cytokine in PD. For instance, induction of chronic expression of IL-1β in adult rat SNpc using a recombinant adenovirus resulted in glial activation, progressive dopaminergic cell death, and akinesia (Ferrari et al., 2006). In a separate study, midbrain dopaminergic neurons were treated with IL-1β which caused significant cell death, comparable to that induced by 6-OHDA (Long-Smith et al., 2009). Although several studies are controversial regarding the neurotoxic role of IL-1β and suggest a more neuroprotective role (Nishimura et al., 2005; Saavedra et al., 2007), the most convincing evidence for IL-1β-induced neurodegeneration comes from genetic and pharmacological studies. Genetic studies observed a significant increase in the microglial IL-1β genotype expression in patients

with PD compared to healthy patients (McGeer et al., 2002). Furthermore, several studies found that the risk of PD doubled in carriers of the homozygous variant of IL-1β position -511 and TNFα position -308 and the risk was tripled for carriers of both variants (Nishimura et al., 2000; Wahner et al., 2007). Pharmacological studies further illustrated that blockade of the IL-1R1 prevented the death of dopaminergic neurons induced by lipopolysachharide (LPS)-treated glial cells, suggesting that IL-1β produced from glial cells contributes to dopaminergic neuronal death (Koprich et al., 2008; Long-Smith et al., 2010). Inhibition of IL-1R1 not only attenuates neurodegeneration, but also reduces PD-associated symptoms, such as dyskinesia, in an animal model (Barnum et al., 2008). Overall, evidence from several types of studies indicates that IL-1β plays a crucial role in the neurodegeneration of PD.

## 2.4 The chemokine family: Overview

Chemokines, which is short for 'chemotactic cytokines', are a family of small (8-14 kDa) cytokines (Jin et al., 2008). Characterization of chemokines have been summarized by Jin et al. (2008) as follows: "The word 'chemokine' is derived from their ability to induce directed chemotaxis on cells that express the appropriate chemokine receptor along a chemical gradient of ligand—known as the chemokine gradient" (Jin et al., 2008). Chemokines are classified into four highly conserved groups based on the position of the first two cysteines adjacent to the N-terminus—C (γ chemokines) CC (β chemokines), CXC (α chemokines), $CX_3C$ (δ chemokines). In the human body, there are currently over 50 chemokines and at least 18 chemokine receptors that have been discovered (Ubogu et al., 2006). Chemokine signaling plays a role in homeostasis,

development, and inflammation. Homeostatic chemokines are chemokines that are produced and secreted without stimulation of the source cell (Luster, 1998; Sallusto et al., 1997). Chemokine secretion from lymph nodes for the chemoattraction of lymphocytes to screen for pathogen invasion is an example of homeostatic chemokines (Jin et al., 2008). Chemokines implicated in development are involved in several activities, including guiding progenitor cells to tissues for cellular maturation (Tiveron et al., 2006). Chemokines that contribute to inflammatory actions are released from a variety of cells in response to a number of agents (i.e., viruses, bacterial infection, pro-inflammatory cytokines). Chemokines mainly function as chemoattractants for leukocytes and other cells from the blood to the site of infection or tissue damage (Jin et al., 2008). In particular, a number of inflammatory chemokines also activate cells to initiate an immune response, an action that can lead to cellular survival or apoptosis (Zlotnik and Yoshie, 2000).

Receptors for chemokines are seven-transmembrane, G-protein-coupled receptors that are found on the surface of peripheral cells, such as leukocytes, as well as brain cells, including astrocytes, microglia, and neurons (Luster, 1998). The actions of neuronal chemokine receptors upon binding by chemokines are known as contributors to neuropathogenesis, by inducing chemotaxis and intracellular calcium transients and amplifying neuronal dysfunction and death (Hirsch and Hunot, 2009; Shimoji et al., 2009; Yasuda et al., 2008).

### 2.4.1 Role of CXCL10/IP-10 in astrocytes and PD

The gene of CXCL10, a 10 kDa α-chemokine, was first identified as an early response gene induced after interferon-γ exposure in a variety of cells, and was therefore

named interferon-inducible protein, IP-10 (Luster et al., 1985; Luster and Ravetch, 1987). CXCL10 is a chemoattractant for activated T cells, monocytes/macrophages (Taub et al., 1993), microglia, and induces astrocyte proliferation (Flynn et al., 2003). In the CNS, astrocytes are the major source of CXCL10 production (Oh et al., 1999). Indeed, CXCL10 expression is significantly induced in TNFα-exposed human A172 astroglial cells (Davis et al., 2007). Furthermore, CXCL10 mRNA and protein expression in astrocytes has been investigated and shown to be markedly increased in the CNS of HIV, AD, and MS subjects (Sui et al., 2004; Sui et al., 2006; Tanuma et al., 2006; Xia et al., 2000). For example, studies demonstrate through post-mortem analysis of neurologically challenged patients (i.e., MS and AD) that CXCL10 is up-regulated compared to healthy patients and the cellular source of this chemokine is astrocytes (Tanuma et al., 2006; Xia et al., 2000). Tanuma and colleagues (2006) further state that astroglial-derived CXCL10 may activate additional astrocytes through an autocrine or paracrine action since the CXCL10 receptor, CXCR3, is found on astrocytes. Eventually, reactive gliosis occurs and is followed by microglia migration and activation, leading to degeneration of neuronal axons (Tanuma et al., 2006). A relatively recent study has shown that CXCL10 is up-regulated in the striatum of the PD *in vivo* MPTP model, yet the cellular source was not established (Kalkonde et al., 2007). Furthermore, studies have indicated detrimental effects of CXCL10 on neuronal cells. For instance, in a HIV study cultured human fetal brain neurons exposed to CXCL10 resulted in calcium dysregulation and activation of caspase-3 and ultimately apoptosis (Sui et al., 2004; Sui et al., 2006). Consequently, strong support for the neurotoxic effects of CXCL10 exists in neurodegenerative

diseases, and thus, more research should be done exploring the modulation of CXCL10 in PD models and patients.

## 2.5 The inducible nitric oxide synthase (iNOS) family

### 2.5.1 The Role of iNOS

Nitric oxide (NO) was first identified in 1980 as the endothelium-derived relaxing factor mediating relaxation of blood vessels (Furchgott and Zawadzki, 1980). Throughout years of research, NO became known as a highly diffusible, bioactive free radical with a short half-life (Steinert et al., 2010). At low concentrations, NO is neuroprotective and mediates physiological signaling (vasodilation and neurotransmission), while at higher concentrations, it mediates immune and inflammatory actions and is neurotoxic (Liberatore et al., 1999). NO is generated by nitric oxide synthase (NOS) via the enzymatic conversion of L-arginine to L-citrulline. There are three NOS genes with distinct tissue locations and functions– neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). The nNOS and eNOS are constitutively expressed and are calcium-dependent enzymes. The inducible form, iNOS, does not require calcium for its activity and is expressed in various cell types including the astroglia and microglia (Steinert et al., 2010). Inducible NOS expression in humans, however, appears to be in astrocytes rather than microglia (Steinert et al., 2010). Nevertheless, all three isoforms of NOS have been reported to contribute to neurodegenerative diseases (Maragakis and Rothstein, 2006). This contribution is demonstrated by NOS production of NO, which reacts with the superoxide radical to ultimately produce reactive nitrogen species (RNS) – peroxynitrite and hydroxyl radical.

NO and its derivatives, RNS, target proteins, DNA, and lipids of cells, resulting in extensive cellular injury and ultimately cell death (Steinert et al., 2010).

### 2.5.2 The importance of iNOS/NO in astrocytes and PD

In the healthy human brain, astrocytes do not express iNOS; however, following trauma or an inflammatory insult, reactive astroglial cells have an up-regulated expression of iNOS (Galea et al., 1992). In fact, the enhanced astroglial iNOS immunoreactivity has been reported in post-mortem brain tissue from patients with MS (Bo et al., 1994), AD (Wallace et al., 1997), and PD (Hunot et al., 1996). Additionally, the iNOS product, NO, is suspected to contribute to a number of neurodegenerative diseases, especially PD (Arimoto and Bing, 2003; Hunot et al., 1996; Iravani et al., 2002; Liu et al., 2002; Okuno et al., 2005). In a PD study, Liberatore et al. (1999) demonstrated a significant up-regulation of iNOS and gliosis after MPTP administration to mice. This group of researchers further observed dopaminergic neurodegeneration following iNOS enhancement, whereas iNOS mutant mice were resistant to the effects of MPTP. A previous study also revealed an astrocytic inflammatory response of an increase in iNOS and NO in an *in vitro* PD-associated gene DJ-1 model (Waak et al., 2009). This increase in NO resulted in an increase of oxidative species which coincided with neuronal apoptosis (Waak et al., 2009). Immunoreactivity analyses of the mesencephalon of post-mortem PD patients further supported the importance of nitric oxide by illustrating a significant increase in glial iNOS levels, which may be neurotoxic to dopaminergic neuronal cells (Hunot et al., 1996). Several other studies have also shown that administration of selective iNOS inhibitors protected nigrostriatal dopaminergic neurons

in a 6-OHDA model (Broom et al., 2011) and in a MPTP mouse model (Kim et al., 2010). Even motor performance in models of PD was affected negatively by NO and improved significantly by inhibition of NOS expression. Padovan-Neto et al. (2009) illustrated this improvement in motor performance when L-DOPA-induced dyskinesia in a rodent model of PD was attenuated after rodents were administrated with NOS inhibitors (Padovan-Neto et al., 2009).

## 2.6 The importance of NF-κB in glia

The transcription factor nuclear factor – κB (NF-κB) was discovered about 25 years ago and observed to bind to the enhancer of the immunoglobulin (Ig) κ light chain gene in B cells (Sen and Baltimore, 1986). Nuclear factor – κB is a ubiquitously expressed transcription factor that compromises five members, p50, p52, p65 (Rel-A), c-Rel, and Rel-B, which share a N-terminal Rel homology domain allowing dimerization, interaction with NF-κB inhibitor IκBα, and binding to DNA at κB sites of target genes (Ghosh and Karin, 2002). Nuclear factor-κB proteins form homo- or hetero-dimers that remain inactive in the cytoplasm by interaction with IκBs (Ghosh and Karin, 2002). IκBs are inhibitory molecules which mask the NF-κB nuclear localization sequence (Malek et al., 2001; Whiteside and Israel, 1997). These molecules are evolutionarily conserved and composed of IκBα, IκBβ, IκBε, IκBγ, IκBζ, Bcl-3, p-105, and p-100 (Whiteside and Israel, 1997). Depending on the stimulus, NF-κB activation can occur through either the classical pathway or the alternative pathway (Figure 6). In the classical or canonical pathway, NF-κB signaling is stimulated by pro-inflammatory cytokines such as TNFα and IL-1β (Bonizzi and Karin, 2004). This stimulation triggers the activation of the IκB kinase (IKK) complex, which is composed of IKKα, IKKβ, and IKKγ/NEMO (NF-κB

essential modulator). IKKβ then phosphorylates IκB proteins on two serine residues, Serine 32 and 36, which then results in the ubiquitination and degradation of IκB by the 26S proteasome (Ghosh and Karin, 2002). The nuclear localization sequence of NF-κB proteins are then unmasked and the proteins are able to translocate to the nucleus and bind to κB sites in the promoter or enhancer regions of target genes and activate their transcription. The alternative pathway or non-canonical pathway involves the upstream kinase NF-κB inducing kinase (NIK), which activates IKK complex, and leads to the phosphylation and processing of p100 to p52. The release of p52-containing dimers then allows for nuclear translocation and DNA binding (Derudder et al., 2003; Xiao et al., 2001). This alternative pathway is thought to play a role in the expression of genes involved in the development and maintenance of secondary lymphoid organs and in adaptive immunity. The pathway operates only upon stimulation by lymphotoxin β, B-cell-activating-factor, or CD40 (Bonizzi and Karin, 2004). However, it is the classical pathway that is involved in the control of innate immunity and inflammation (Baud and Karin, 2001; Bonizzi and Karin, 2004; Ghosh and Karin, 2002) and in the regulation of the production of several pro-inflammatory mediators including cytokines (TNFα), chemokines (monocyte chemotactic protein-1), enzymes (cyclooxygenase-2), and nitric oxide.

Several reports suggest a critical role for NF-κB in the manifestation of PD (Aoki et al., 2009; Niranjan et al., 2010). In support of the role of NF-κB in astrocytes in PD, studies have shown an enhancement of active NF-κB levels in astrocytes in the presence of PD-associated neurotoxin MPTP (Aoki et al., 2009; Niranjan et al., 2010). Both groups of researchers demonstrate an increase in NF-κB translocation, with nuclear p65

levels significantly increased in astroglial cells after MPTP treatment. A 70-fold increase in the nuclear translocation of NF-κB compared to age-matched healthy controls is also seen in substantia nigra dopaminergic neurons of post-mortem PD patients (Hunot et al., 1997). Therefore, these studies suggest a critical role for NF-κB, especially through the classical pathway, in PD.

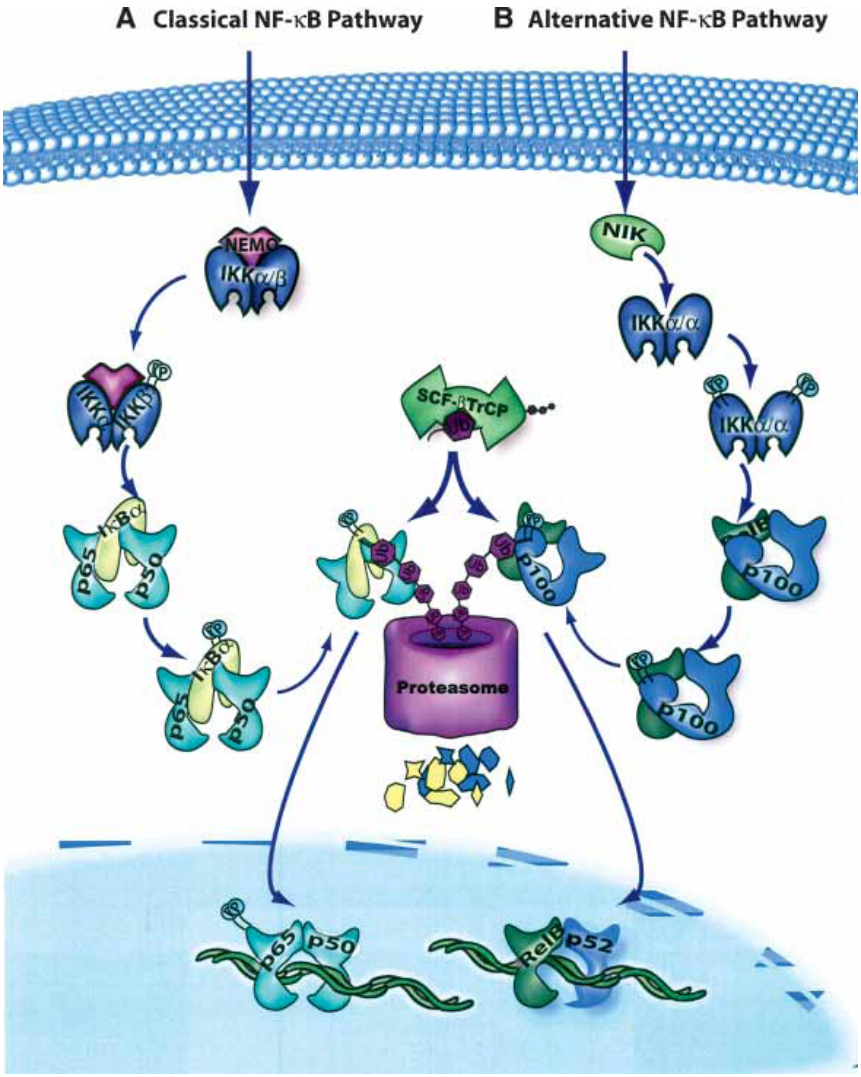


**Figure 6. Classical and alternative pathways of NF-κB activation.** A model depicting the two signaling pathways to NF-κB. (*A*) The classical pathway is mediated by IKKβ and leads to phosphorylation of IκB. Inputs for the classical pathway include TNFR1/2, TCR and BCR, and TLR/IL-1R. (*B*) The alternative pathway involves NIK activation of IKKα and leads to the phosphorylation and processing of p100, generating p52-RelB heterodimers. Input signals for the alternative pathway include LTβR, BAFFR, and CD40R. Image copied from *Genes and Development* with permission (Hayden and Ghosh, 2004).

### 2.6.1 The importance of NF-κB in CXCL10 production in astrocytes

The signaling pathway responsible for the induction of CXCL10 is important to examine in the pathogenesis of neuronal diseases. CXCL10 induction was found to be regulated transcriptionally by the activation of NF-κB (Williams et al., 2009b). Interestingly, the CXCL10 promoter NF-κB binding site has exclusive affinity to p50/p65 heterodimers (Ohmori and Hamilton, 1995). We have established, through the use of specific NF-κB inhibitors, a signaling pathway responsible for the production of CXCL10 in astroglial is NF-κB (Davis et al., 2007). In fact, the inactivation of astroglial NF-κB resulted in down-regulation of the production of CXCL10 expression, and ultimately led to a neuroprotective effect on retinal neurons following ischemic injury (Dvoriantchikova et al., 2009). In another study, inactivation of NF-κB and reduced CXCL10 resulted in a faster recovery after spinal cord injury in transgenic mice compared to non-transgenic mice (Brambilla et al., 2005). Furthermore, human astrocytes co-exposed to HIV-1 Tat and proinflammatory cytokines resulted in the induction of CXCL10 at both the RNA and protein level (Williams et al., 2009b).

### 2.6.2 The importance of NF-κB in iNOS/NO induction in astrocytes and PD

The involvement of NF-κB in the production of NO has been illustrated in several studies. First, the human iNOS gene contains eight NF-κB binding sites (Taylor et al., 1998). Secondly, Davis and colleagues (2005) illustrated the dependency of iNOS expression in astroglial cells on NF-κB by the use of several NF-κB inhibitors (MG-132, helenalin, BAY 11-7082). In an *in vitro* PD model, mesencephalic cells exposed to manganese demonstrated increased NO generation during the activation of NF-κB, which

was blocked by NF-κB inhibitor (SN50) (Prabhakaran et al., 2011). Furthermore, in a PD animal model, administration of paraquat intraperitoneally resulted in the induction of nitric oxide and production of lipid peroxidation in the striatum (Gupta et al., 2010). However, this induction of iNOS mRNA and protein and expression of lipid peroxidation were significantly attenuated in the animal models by the NF-κB inhibitor pyrrolidine dithiocarbamate (Gupta et al., 2010). A recent study explored the role of astroglial NF-κB in iNOS induction and NOS2 expression in an *in vivo* and *in vitro* PD model, respectively (Miller et al., 2011). This study by Miller et al. (2011) demonstrated that MPTP-exposed astrocytes had colocalized expression of iNOS and NF-κB, with an increased protein nitration in nigral dopaminergic neurons. Inhibition of NF-κB in astroyctes by mutant IκBα suppressed expression of NOS2 and protected co-cultured neurons from astrocyte-mediated apoptosis (Miller et al., 2011). Consequently, NF-κB has been implicated in the induction of iNOS in PD pathogenesis, yet further studies are required to examine the role of astroglial NF-κB on iNOS induction in PD.

Astrocytes are the major source of NF-κB-induced CXCL10 and iNOS in the CNS (Ohmori and Hamilton, 1995; Taylor et al., 1998). This chemokine and enzyme potentially exacerbate an inflammatory attack by mediating chemotaxis and producing reactive nitrogen species at the site of insult, respectively. Therefore, studies should be done on astrocytes to elucidate the role of these inflammatory factors (NF-κB, CXCL10, iNOS) on Parkinson's disease neuropathogenesis.

## 2.7 Neuromelanin

### 2.7.1 The role of NM in normal brain

In the human brain, the neurotransmitter dopamine (DA) has been implicated in a number of roles, including voluntary movement, reward, motivation, and mood (Bromberg-Martin et al., 2010). Tyrosine hydroxylase (TH) produces L-3,4-dihydroxyphenylalanine (DOPA), which is then rapidly converted to DA by aromatic amino acid decarboxylase (AADC) (Sidhu et al., 2004). Cytosolic DA can have several destinations, including accumulation in synaptic vesicles via uptake by vesicular monoamine transporter 2 (VMAT2). Dopamine can also be metabolized by monoamine oxidase (MAO) into 3,4-Dihydroxyphenylacetic acid (DOPAC). Cytosolic DA can also be released from the cell via reverse transport through the dopamine transporter (DAT) (Sulzer, 2007; Zucca et al., 2004). If these processes are insufficient to control the levels of cytosolic DA, excess cytosolic DA can be easily oxidized. Dopamine oxidation can occur via two major pathways: auto-oxidized or enzymatic oxidation, both of which produce dopamine-quinones and ROS in the cytosol. Oxidized dopamines (oxDA) are highly reactive and exert cytotoxicity in DA neurons and surrounding neural cells (Asanuma et al., 2003; Miyazaki and Asanuma, 2008). Oxidized DA reacts with cysteine residues on proteins to form DA-Cys-protein products, which can be phagocytized in autophagic vacuoles and become double-membraned NM granules over time (Fedorow et al., 2005; Zucca et al., 2004). Neuromelanin, meaning 'black' substance in neurons, is what causes the dark pigmentation of certain neurons and is found at its highest levels in the SNpc and the locus coeruleus (LC) (Graham, 1979; Zecca et al., 2004). In the human brain, NM first appears in the SNpc around the third year of life (Fenichel and Bazelon, 1968) and accumulates linearly with age, reaching concentrations of 2.3-3.7 µg/mg in the SNpc of healthy subjects aged 50-90 years old (Zecca et al., 2002).

It has been suggested that the synthesis of NM plays a protective role within the cell (Fedorow et al., 2005; Zecca et al., 2003; Zucca et al., 2004). Neuromelanin prevents the accumulation of toxic cytosolic DA derivatives by incorporating them into the polymer (Sulzer et al., 2000). Neuromelanin may also play a protective role by acting as a 'black hole' capable of chelating redox active metals (iron, zinc, copper), toxic metals (mercury and lead), and a wide variety of toxic compounds (MPP+) (D'Amato et al., 1986; Enochs et al., 1994; Zecca et al., 2002), indicating that it could be a high capacity storage system that prevents neuronal damage.

### 2.7.2 The role of NM in PD brain

Although brain regions other than the pigmented SNpc are involved in the pathology of PD, the loss of NM-containing neuronal cells in this area represents a cardinal pathologic diagnostic criterion for the disease. Specifically, NM levels in PD patients were 1.2-1.5 µg/mg in the SNpc, which are less than half of age-matched controls (Zecca et al., 2002). In fact, analysis of PD midbrain shows that dopaminergic neurons of the SN containing NM are predominately more vulnerable to loss than the unpigmented neurons (Gibb, 1992; Kastner et al., 1992). It has been suggested that the pigment itself increases the vulnerability of SN neurons and leads to neurodegeneration (Offen et al., 1997). The observed decrease of NM in the SNpc of PD patients further supports the loss of the pigmented neurons during PD (Gibb and Lees, 1991; Pakkenberg et al., 1991). Others have stated that the level of NM actually decreases in surviving neurons of the SN (Kastner et al., 1992; Mann and Yates, 1983) due to a reduced biosynthesis, increased degradation, higher vulnerability of heavily pigmented neurons, or a combination of these mechanisms.

In contrast to NM's cytoprotective roles, there is also evidence of this polymer exhibiting a cytotoxic role and, hence, explaining the death of pigmented neuronal cells in PD brains. Studies have reported that when free neuronal iron increases and saturates the chelating sites of NM, NM could lead to an increased production of free radicals, such as hydrogen peroxide and hydroxyl radicals (Double et al., 2002; Zareba et al., 1995). As hydrogen peroxide and hydroxyl radicals degrade the integrity of NM, cytotoxic redox active metal ions will be released from NM and could accelerate SNpc neuronal death by exacerbating oxidative stress (Double et al., 2002; Enochs et al., 1994). The release of NM from these dying neurons could trigger a vicious cycle of neuroinflammation and neurodegeneration. If the anti-oxidative defense mechanism is overwhelmed and the cumulative stress is severe enough, exacerbation of neuronal depletion could increase the onset or symptoms of PD (Tansey et al., 2007).

#### 2.7.2.1 The effect of NM on proinflammatory factors in PD

In subjects with juvenile (Ishikawa and Takahashi, 1998), idiopathic, and MPTP-induced parkinsonism (Langston et al., 1999), neuropathological examinations have demonstrated the presence of insoluble extracellular NM granules, which arise following SNpc cell death and remain in the EC space in large amounts for long periods of time (months, years). In a patient with MPTP-induced parkinsonism, extraneuronal melanin was found with activated microglial cells and gliosis even 12 years after exposure to the neurotoxin (Langston et al., 1999). An *in vitro* study demonstrated the effect of human NM on rat microglia and illustrated NM's ability to trigger microgliosis, microglial chemotaxis, and microglial activation with subsequent release of proinflammatory and neurotoxic mediators such as NO, TNFα, and IL-6 (Wilms et al., 2003; Zhang et al.,

2011). Addition of NM to microglial cells also activated the NF-κB and p38 MAPK signaling pathways (Wilms et al., 2003). Further support for glial activation by extraneuronal NM was shown in the SNpc of post-mortem patients of PD and MPTP-intoxication, where NM deposits were found to be phagocytosed by microglia (Zucca et al., 2004) and to induce glia activation (Zhang et al., 2011). Due to the lack of research on NM effects on astrocytes, the present study investigated the pro-inflammatory factors triggered by exogenous NM and its pathogenic mechanism in human astrocytoma cells. The findings from these studies may provide insights into the inflammatory mechanism of PD caused by the extraneuronal deposits of NM.

## 2.8 Alpha-synuclein

### 2.8.1 The role of α-synuclein in normal brain

Alpha-synuclein belongs to the synuclein family along with β- and γ-synuclein (George, 2002; Ueda et al., 1993), which have been described only in vertebrates. Alpha-synuclein is predominately expressed in the brain neocortex, hippocampus, striatum, thalamus, and cerebellum and is the only synuclein known to be implicated in disease (Iwai et al., 1995). Human α-synuclein is a small acidic protein (14 kDa) composed of 140 amino acid residues and predominately localized in presynaptic terminals in the CNS, where it loosely associates with synaptic vesicles (Norris et al., 2004). Alpha-synuclein was first identified as the precursor of the non-Aβ component peptide, which is present in extracellular amyloid plaques of AD patients (Iwai et al., 1995; Ueda et al., 1993). Although the physiological roles of α-synuclein are not fully understood, numerous studies demonstrate the role of α-synuclein in DA neurotransmission regulation (Dev et al., 2003; Sidhu et al., 2004). Studies demonstrate α-synuclein's role

by its effect on neuronal plasticity (Clayton and George, 1998), regulation of neuronal membrane trafficking via vesicle budding or turnover (Chen et al., 1997), regulation of the size of synaptic vesicle pools in neurons (Murphy et al., 2000), inhibition of TH activity (Perez et al., 2002), and regulation of the number of plasma membrane DATs (Lee et al., 2001).

### 2.8.2 The role of α-synuclein in PD brain

The implication of α-synuclein's role in the pathophysiological process of PD is still far from obvious; however, a prerequisite of α-synuclein neuropathy is its oligomerization into soluble protofibrils followed by their coalescence into insoluble fibrils, and accumulation into Lewy bodies (LB) (Lee and Lee, 2002; Maries et al., 2003). Several conditions induce α-synuclein aggregation, including α-synuclein mutations (A30P, A53T, E46K), over-expression, and exposure to neurotoxins (MPTP, rotenone) and oxidative factors (free iron, oxidized dopamine) (Dev et al., 2003; Lee, 2008). Once aggregation is initiated, the bioavailability of α-synuclein is greatly diminished such that normal physiological functions regulated by α-synuclein may be severely compromised, which in turn, could further worsen the initial cellular insult (Sidhu et al., 2004) (see Figure 7).

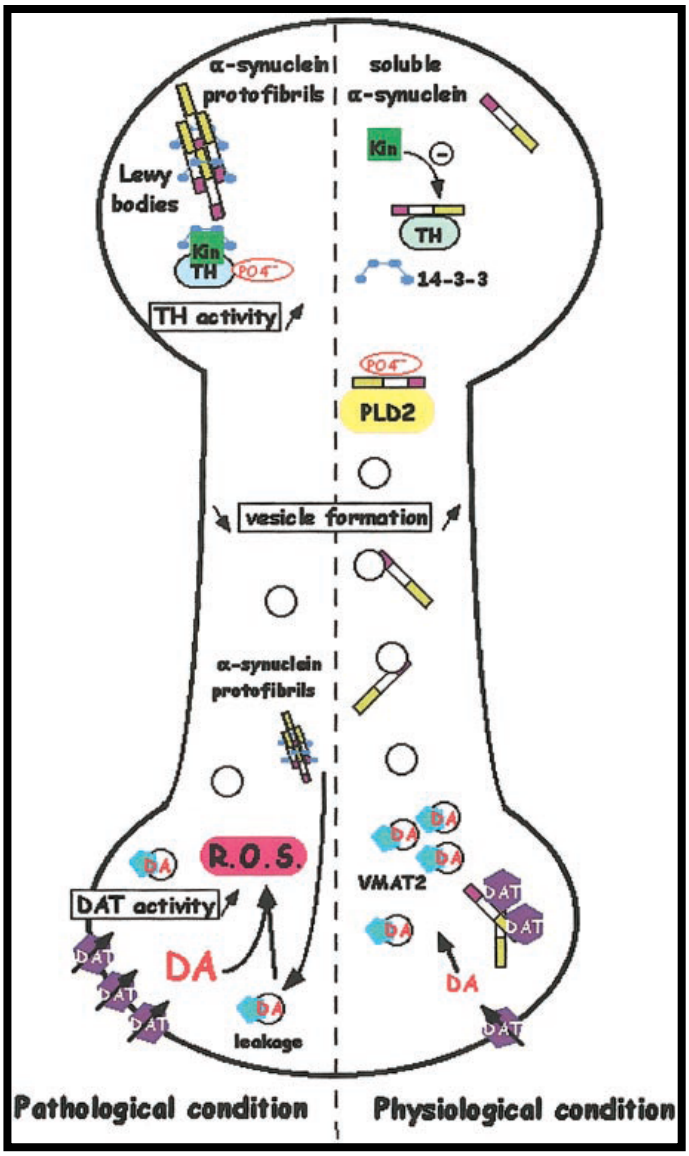


**Figure7. Overview of the consequences of α-synuclein aggregation that turn it from a neuroprotective to a toxic molecule.** Under physiological conditions (right side of diagram), α-synuclein is able to lower TH activity by blocking interactions with 14-3-3 proteins and various kinases, which are required to trigger a high TH activity. Alpha-synuclein also contributes to a normal rate of synaptic vesicle formation, which leads to an efficient clearance of cytoplasmic dopamine through VMAT2. Normal amounts of soluble α-synuclein at the nerve terminals helps to hold the dopamine transporter (DAT) into a cytoplasmic compartment, preventing overload of extracellular dopamine into the neuronal cytoplasm. Overall, α-synuclein tends to decrease the amount of free dopamine inside neurons, and its possible transformation into a reactive and highly toxic molecule. In contrast, under pathological conditions (left side of the diagram), lowering the amount of soluble α-synuclein tends to increase free cytoplasmic dopamine and the formation of reactive oxygen species (ROS). Formation of protofibrils or aggregates and LBs diminishes the availability of the physiological forms of α-synuclein, favoring an increase in TH and DAT, and diminishing vesicles formation and neuronal plasticity. Image copied from *The FASEB Journal* with permission (Sidhu et al., 2004)

The link between α-synuclein and PD, including idiopathic PD, was solidified by the finding that fibrillar aggregates of this protein are the main components of the pathological hallmark of PD: LB and Lewy neurites (Spillantini et al., 1998). It is widely accepted that during the course of PD, α-synuclein is not only accumulated in cytosolic space and vesicles, leading to a possible internal effect on SNpc neurons, but is also released into the extracellular space. Furthermore, the presence of α-synuclein has been observed in the CSF and blood of Parkinson's disease patients (El-Agnaf et al., 2003). The presence of α-synuclein in the extracellular space can thereby gain access to the glial environment. More specifically, α-synuclein released from neurons induces glial activation, resulting in production of neurotoxic molecules (superoxide anions, intracellular reactive oxygen species, prostaglandin E2, intercellular adhesion molecule-1, and IL-6) which lead to neuroinflammation and neurodegeneration of dopaminergic neurons (Kahle et al., 2000; Klegeris et al., 2006; Xu et al., 2002; Zhang et al., 2005; Zhou et al., 2002). Because of this action, there has long been speculation that α-synuclein underlies PD pathogenesis.

#### 2.8.2.1 The effect of α-synuclein on proinflammatory factors in PD

Numerous *in vitro* and *in vivo* studies have been carried out to elucidate the neuroinflammatory and neurodegenerative effects of α-synuclein leading to neuronal dysfunction and death. These effects are seen in experimental PD models using mutated, overly-expressed, and more recently, extracellular α-synuclein. Studies examining the effects of overexpressed or mutated α-synuclein in cells and transgenic animals resulted in mitochondrial dysfunction, which led to formation of inclusion bodies, promotion of oxidative stress, and ultimately neuronal loss (Hsu et al., 2000; Kirik et al., 2002; Klein et

al., 2002; Masliah et al., 2000; Zhou et al., 2002). Some transgenic models of overexpressed α-synuclein also demonstrated an increase in the production or expression of several proinflammatory molecules, including TNFα, IL-1β, NO, and CCR3 in microglia which then led to neurodegeneration when exposed to neurons (Gao et al., 2008; Su et al., 2008a; Su et al., 2009). Only a few studies have actually examined the effects of extracellular α-synuclein on glial cells. For example, extracellular α-synuclein was shown to activate microglial cells and to up-regulate several pro-inflammatory molecules, such as NO, IL-1β, IL-6, COX-2, $CX_3CR1$, NOS2, and NADPH oxidase with production of ROS (Su et al., 2008a; Su et al., 2009; Zhang et al., 2005). The effect of α-synuclein on microglial activation resulted in cytotoxicity of a human neuroblastoma cell line (El-Agnaf et al., 1998) and primary mesencephalic dopaminergic neuron-glial system (Zhang et al., 2005). Klegeris et al. (2006) studied the effects of extracellular α-synuclein on human astroyctes and astrocytoma cells and demonstrated an induction of ICAM-1 and IL-6 (Klegeris et al., 2006). The current study describes the effect of exogenous α-synuclein on pro-inflammatory factors CXCL10 and iNOS and the pathogenic mechanism in human astrocytoma cells. These findings may provide insights into the inflammatory mechanism of PD caused by the abnormal accumulation of α-synuclein aggregates.

## 2.9 Understanding the effects of PD-associated molecules in human astroglial cells

In this dissertation, the cell-dependent effects of NM and α-synuclein are investigated through cellular and molecular characterizations. Examining the expression of inflammatory factors by these PD-associated molecules will provide a better

understanding of the role of the molecules in PD. Although numerous effectors such as environmental toxins and genetic factors can initiate SNpc and striatum neuronal damage, this introduction has described the release of NM and α-synuclein from neurons inducing the production and release of neurotoxic glial factors (Kahle et al., 2000; Klegeris et al., 2006; Wilms et al., 2003; Xu et al., 2002; Zhang et al., 2005; Zhou et al., 2002). These glial factors, in turn, provoke further neuronal death and further release of NM and α-synuclein, thus leading to a vicious cycle of neuroinflammation and neurodegeneration. This process has recently been examined in rodent and human microglial cells, yet still remains to be explored in astroglial cells. Therefore, it is important to elucidate the effects of NM and α-synuclein on human derived astroglial cells.

## 2.10 Hypothesis and Specific Aims

The hypothesis for this dissertation is presented two-fold:

**1 - Neuromelanin and α-synuclein, separately, increase cytokine-induced NO and CXCL10 expression in A172 astroglial cells.**

The first hypothesis was examined through experiments within the following specific aims:

a) Determine time and dose-dependent effects of NM and α-synuclein (separately) on NO and chemokine protein expression in TNFα/IL-1β-exposed astrocytes.

b) Determine the role of NM and α-synuclein on iNOS and CXCL10 mRNA expression, respectively.

c) Determine the effect of α-synuclein on CXCL10 mRNA stability.

**2 - NM and α-synuclein, separately, increase the effect of cytokine-induced NF-κB activation in A172 cells.**

The second hypothesis was examined through experiments within the following specific aims:

a) Determine the role of NM and α-synuclein on NF-κB-DNA binding.

b) Determine the effect of α-synuclein on I-κBα phosphorylation.

The goal of this study is to examine the human astroglial response under protein-inflammation conditions with NM and α-synuclein. Furthermore and most importantly, the mediators (iNOS, CXCL10, NF-κB) examined in this dissertation have been shown to play crucial roles in the process of inflammation and neuronal death. Consequently, valuable information is gained by understanding the actions of NM and α-synuclein on the induction of neurotoxic molecules and the signaling pathway in a cytokine-exposed human astroglial condition. This dissertation is the first study that examines the effects of NM and α-synuclein on human astroglial cells, ultimately advancing the development for therapeutic strategies to combat PD-associated neuroinflammation.

CHAPTER III

# RESEARCH DESIGN AND METHODOLOGY

## 3.1 Cell cultures

### 3.1.1 Human A172 Astrocytoma cells

Human A172 cells (ATCC #CRL-1620) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine, 10% fetal bovine serum, 1% nonessential amino acids, 50 U/ml penicillin, 0.05 mg/ml streptomycin and 2 μg/ml amphotericin B. Proinflammatory pathways in these human brain cells (ATCC #CRL-1620; American Type Culture Collection, Manassas, VA) have been previously characterized (Davis et al., 2002; Davis and Syapin, 2004a; Davis and Syapin, 2004b) and used in various pharmacology- and neurochemistry-based studies (Guthikonda et al., 1998; Kubota et al., 2001; Zaheer et al., 1995).

### 3.1.2 Human SK-N-SH Neuronal cells

The human SK-N-SH neuronal cells (ATCC #HTB-11) were maintained in RPMI 1640 with 10% fetal bovine serum, 50 U/ml penicillin, 0.05 mg/ml streptomycin, and 2 μg/ml amphotericin B as previously described (Wallace et al., 2006).

### 3.1.3 Human CHME-5 Microglial cells

Human CHME-5 microglial cell line (developed by Dr. Marc Tardieu, Paris, France (Janabi et al., 1995) was a kind gift from Dr. Pierre Talbot, Quebec, Canada. CHME-5 cells were maintained in DMEM containing 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, 0.05 mg/ml streptomycin, and 2 μg/ml amphotericin B. All cultures were maintained in a humidified incubator at 37°C, 5% $CO_2$ and 95% air with the culture medium changed every 48–72 hour. Experimental cultures were seeded at a cell density to provide 80–90% confluence at the time of treatment.

## 3.2 Preparation of PD-associated molecules

### 3.2.1 Oxidized DA Preparation

Dopamine was dissolved in serum-free DMEM (containing iron) to obtain a 10 mM solution followed by filter sterilization. One ml of solution was transferred to a microcentrifuge tube, covered with aluminum foil to avoid light exposure, and vortexed gently for 1 h. Oxidized DA was then diluted in culture medium to the required concentration (3.7-600 μM). (Methodology obtained from Douglas Walker, PhD at Sun Health Research Institute in Sun City, Arizona)

### 3.2.2 Neuromelanin Preparation

Neuromelanin was isolated as previously described (Zecca et al., 2008) from substantia nigra samples obtained during autopsies of subjects who died without any known neurological or psychiatric disorders. The purity of the NM was assessed by elemental analysis, amino acid analysis and electron paramagnetic resonance spectroscopy as previously described (Zecca et al., 2000; Zecca et al., 2002). Stock NM

was resuspended in 0.5 ml ethanol, agitated gently, and ethanol was evaporated under nitrogen flow. NM was then suspended in water (0.5 mg/ml). The sample tube was wrapped in aluminum foil to avoid light exposure, sonicated for 10-20 minutes followed by gently agitation for 3-4 days at room temperature. After the initial 15 hours of agitation, granules were broken up with a glass Pasteur pipette tip, sonicated for 10 minutes, and returned to shaker. For further fragmentation of granules during the 4 day shaking, sonication was done for 10 minutes every 15-20 hours. Aliquots of 180 µl were stored at -80°C in sterile 1.5ml centrifuge tubes. Upon thawing, samples were sonicated for 20 minutes, followed by 24 hour of agitation to obtain a homogenous suspension. NM suspension was diluted to desired concentrations (0.02-15 µg/ml) in serum-free culture medium and incubated at room temperature for 2 hour before adding to cell cultures. The biological relevance of this concentration range of NM was based on the *in situ* concentrations found in the substantia nigra of normal subjects (Zecca et al., 2001).

### 3.2.3 Alpha-synuclein Preparation

Purified human α-synuclein was obtained from r-Peptide (cat.# S-1001). Upon arrival, α-synuclein power was resuspended in 0.5 ml of sterile water to obtain a concentration of 1 mg/ml. Aliquots of 10 µl were stored at -80°C in sterile 1.5 ml centrifuge tubes. Upon thawing, samples were diluted with 900 µl of sterile PBS to obtain a stock concentration of 690 nM. Tubes were agitated ("aged") for either 7 or 14 days at 37°C for formation of an inflammatory-inducing conformation, followed by dilution to desired concentrations in serum free medium and exposure to appropriate cell wells. The concentration and duration of α-synuclein is provided in the results section of each experiment.

### 3.3 MTT test for cell viability

To assess cell viability, the MTT assay was performed according to a modified version of a previously described procedure (Carmichael et al., 1987). Cell viability in response to treatment was determined colorimetrically by measuring the reduction of tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yI)-2,5 diphenyl Tetrazolium Bromide, Table 1) to insoluble formazan by mitochondrial dehydrogenase. Following the appropriate length of stimulation, culture media was replaced with fresh serum free medium containing 0.55 mg/ml MTT and returned to humidified incubator ($37^{o}$C) for 45 minutes. Media was aspirated and cells will be dissolved in 1 ml dimethyl sulphoxide (DMSO, ACS grade Sigma D-8779) to dissolve the developed formazan for a minimum of ten minutes on a rocker at room temperature. Absorbance was measured at 492 nm using a BIO-TEK HT spectrophotometer. The absorbance values from wells containing drug treated cells were compared to the values from untreated control cells to determine the viability of cells in each treatment group.

**Table 1. Preparation of MTT solution**

| | |
|---|---|
| MTT stock solution | Dissolve 100 mg MTT (Sigma M-5655) in 20 ml sterile PBS, Sterile filter in 50 ml centrifuge tube, Wrap with aluminum foil and store at $4^{o}$C |

### 3.4 Analysis of iNOS expression

As an index of iNOS induction, nitrite accumulation in the culture media was determined spectrophotometrically using the Griess reagent (Davis et al., 2002). First, standard curve was prepared in the same media as used in experiments. Quantitative

standards were generated by serial diluting sodium nitrite into culture medium. To prepare standard curve (Table 2), one ml of medium was pipetted into test tubes labeled #1-6. Two ml of medium was pipetted into tube #7. The standard was pipetted into tube #7 at a volume of 16 µl and was mixed gently on vortex machine. One ml from tube #7 was transferred into tube #6 and gently mixed on vortex machine. This transfer was repeated in 1:1 dilutions until tube #2 had 2ml. Tube #1 did not contain the standard since it was used as the blank. Therefore, the nitrite concentrations in 0.1 ml were:

Tube #1 = 0 nmol
Tube #2 = 0.25 nmol
Tube #3 = 0.5 nmol
Tube #4 = 1.0 nmol
Tube #5 = 2 nmol
Tube #6 = 4 nmol
Tube #7 = 8 nmol

Duplicate 0.1 ml aliquots from these tubes were pipeted into appropriate wells of a 96-well Falcon plate (first two columns). After this, duplicate 0.1 ml aliquots from unknown samples were pipeted into appropriate wells of a 96-well Falcon plate. The Griess reagent was prepared for addition to the wells containing standards and samples. Eleven ml of Griess reagent was prepared for each 96-well plate (5.5 ml of Solution 1 + 5.5 ml of Solution 2, Table 2). One hundred µl of the Griess reagent was added to each well and plate was read after 5 minutes. Absorbance was read at 546 nm using a BIO-TEK HT spectrophotometer. Total cell protein (see section 3.6.1) was calculated to normalize data as nanomoles of nitrite per milligram of protein.

**Table 2. Preparation of Nitrite assay standard and solutions**

| | |
|---|---|
| Standard | 10 mM sodium nitrite in distilled water (dissolve 0.069 g of sodium nitrite into 85 ml distilled water, bring up to 100 ml with water after resuspension) |
| Solution 1 | 0.1% (W/V) naphthylethylenediamine dihydrochloride in distilled water (dissolve 0.5 g into 450 ml distilled water, bring up to 500 ml with water after resuspension) |
| Solution 2 | 1% (W/V) sulfanilaminde in 5% (V/V) concentrated phosphoric acid (carefully add 25 ml concentrated phosphoric acid into 400 ml distilled water, dissolve 5 g sulfanilamide, bring up to 500 ml with water after resuspension) |

## 3.5 Quantitative analysis of mRNA expression

### 3.5.1 RNA isolation

Total RNA was isolated from astrocytic cells with TRIzol reagent (Invitrogen) using manufacturer's instructions. Briefly, cells were lysed directly in the culture dish by adding 700 µl of TRIzol and passing the cell lysate several times through a pipette. The homogenized samples were transferred into eppendorf tubes (per one sample) and incubated for 5 minutes at room temperature (RT) to permit the complete dissociation of nucleoprotein complexes. To separate RNA from DNA and protein, 200 µl of chlorform was added to each tube and shaken vigorously by hand for 15 seconds. Samples were incubated for 2-3 minutes at RT, followed by centrifuged for 15 minutes at 12,000 × g ($4^{o}$C). Following centrifugation, the mixture separated into a lower red, phenol-chloroform phase (protein), an interphase (DNA), and a colorless upper aqueous phase (RNA). Since the RNA remains exclusively in the aqueous phase, the aqueous phase was transferred 100 µl at a time into a fresh eppendorf tube. The RNA was precipitated by mixing with 700 µl (1:1 ratio of TRIzol and isopropyl alcohol) isopropyl alcohol.

Samples were incubated at RT for 10 minutes and centrifuged for 10 minutes at 12,000 × g for 10 minutes at 4$^{o}$C. The RNA precipitate was observed as a white pellet on the bottom of tubes. The supernatant was removed and the RNA was washed twice to remove any residual compounds. Supernatant was washed with 75% ethanol (1 ml/tube), and tubes were vortexed and centrifuged at 7,400 × g for 5 minutes at 4$^{o}$C. These last set of steps were repeated once more. At the end of this procedure, the RNA pellet was dried at RT for 5 minutes to remove remaining ethanol. RNA samples were dissolved in 30 µl RNase-free water and incubated for 10 minutes at 55-60$^{o}$C in water bath. The tubes were vortexed and centrifuged. RNA samples were quantitated on a ND-1000 (NanoDrop Technologies) and their integrity was determined by denaturing agarose gel electrophoresis. To evaluate the quantity and purity of the extracted RNA, spectrophotometric readings were obtained at wavelengths of 260 nm and 280 nm. A reading at wavelength of 260 nm allowed for calculation of the concentration of RNA in the sample. An optical density of 1 corresponded to approximately 40 µg/ml of single-stranded RNA. The ratio of the absorbance at 260 nm and 230 nm (A260/A230) provided information on chemical (i.e. ethanol) contamination. The ratio of the absorbance at 260 nm and 280 nm (A260/A280) provided an estimate of the purity of RNA. Pure preparations of RNA displayed an A260/A280 ratio of between 1.8 and 2.0. Contamination with proteins or phenol resulted in a lower A260/A280 ratio and hampered the accurate quantification of RNA. RNA integrity was determined with ~1 µg of total RNA on a 1% agarose gel. These RNA samples were combined with loading buffer (1:1 ratio, Ambion) and incubated for 30 minutes at 50$^{o}$C in a water bath prior to gel electrophoresis. 18s and 28s rRNA bands were visual determinants of the RNA

quality and integrity. Lack of smearing or clumping of bands demonstrated non-degraded RNA.

### 3.5.2 DNA elimination and cDNA synthesis

One µg RNA from each sample was treated with DNase (Invitrogen) to eliminate any genomic contamination and then reverse-transcribed to cDNA, using High Capacity cDNA Synthesis kit (Applied Biosystems), following the kit's instructions. A 2× reverse transcription master mix was prepared using nuclease-free water, 10× RT buffer, 25× dNTP mix, 10× Random Primers, RTase, and RNase inhibitor. cDNA was then synthesized in a thermal cycler using the following cycling parameters: 10 minutes at 25$^{o}$C, 120 minutes at 37$^{o}$C, followed by 5 minutes at 85$^{o}$C. Freshly synthesized cDNA was directly used for PCR quantitative application or stored at 4$^{o}$C until PCR was performed. To check accurate cDNA synthesis, some cDNA samples were examined on a 2% agarose gel composed of TE buffer and Ethitium Bromide. Four µl of cDNA were combined with one µl of Blue Juice Gel Loading buffer (Invitrogen) and examined on the gel to visualize cDNA synthesis.

### 3.5.3 Quantitative real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR)

Quantitative real-time RT-PCR was performed using SYBR Green chemistry on an ABI StepOne Real-Time PCR System (Applied Biosystems). PCR reactions were performed using 100 ng of cDNA as template, CXCL10 target gene and GAPDH endogenous control specific primers, Power SyberGreen, and DEPC-treated water to obtain a final volume of 20 µl. The PCR primers were purchased from www.realtimeprimers.com and Invitrogen (Table 3) and were used at a concentration of

100 nM per reaction. Power SYBR Green master mix (Applied Biosystems) was used for the SYBR Green PCR reactions. Standard SYBR Green protocols were followed for PCR amplification. Thermal cycling conditions were 95°C for 10 minutes, followed by 45 additional cycles of 95°C for 15 seconds, 63°C for 15 seconds, and 72°°C for 20 seconds.

To assure amplification of a single product, a melt curve analysis was performed and analyzed for all the SYBR Green real-time PCR reactions. Reaction products were checked on 2% agarose gel to confirm the correct amplicon size generated following PCR amplification of each target. Evaluated expression of several endogenous genes as candidates for control genes for relative expression analysis is presented in Table 3. Expression of GAPDH was determined to be an appropriate endogenous control for gene expression studies. Primer sequence information for target gene and endogenous controls is presented in Table 3.

**Table 3: Primer pairs for SYBR green real-time RT-PCR (realtimeprimers.com and Invitrogen)**

| Target Gene Name | Forward and Reverse primer sequence(5'-3') | Primer Tm | Amplicon size | Gene Accession |
|---|---|---|---|---|
| **CXCL10** | Fp:AACCTCCAGTCTCAGCACCATGAA<br>Rp:AGGTACAGCGTACGGTTCTAGAGAG | 78°C | 112bp | NC_000004.11 |
| **HPRT1** | Fp: GCTGACCTGCTGGATTACAT<br>Rp:TTGGGGCTGTACTGCTTAAC | 58°C | 242bp | NM_013556.1 |
| **18S** | Fp:TTCGAACGTCTGCCCTATCAA<br>Rp:GATGTGGTAGCCGTTTCTCAGG | 85°C | 118bp | NT_167214.1 |
| **PPIA** | Fp:AGCTCTGAGCACTGGAGAGA<br>Rp:GCCAGGACCTGTATGCTTTA | 58°C | 178bp | AK028210 |
| **GAPDH** | Fp:GAGTCAACGGATTTGGTCGT<br>Rp:TTGATTTTGGAGGGATCTCG | 80°C | 110bp | NM_007393.1 |

CXCL10: Interferon-γ-inducible protein 10kDa
HPRT1: Hypoxanthinephophoribosyltransferase-1
PPIA: Peptidylprolyl isomerase A
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

To ensure PCR efficiency, several validation experiments were conducted to check the quality of the procedure. RNA standard curve was prepared for all the targets to ensure 90-100% RT efficiency of the reaction for the target. To assure that amplification efficiency fell between 95-100%, experiments were conducted for the set of primers using serial-fold dilutions of cDNA in comparison with the endogenous control. Also, 2 µl of the PCR product was examined on 2% agarose gel containing 1 µl ethidium bromide to visualize the 10 kDa fragment. Additionally, all PCR experiments were performed in duplicates.

### 3.5.4 mRNA stability assay

The measurement of mRNA stability was performed in A172 cells according to previously published methods (Maier et al., 2007). Prior to evaluating the effects of α-synuclein on mRNA stability, the inhibitory effect of actinomycin D was examined in A172 cells. To examine this, treated cells were exposed to 3, 10, or 30 µg/ml actinomycin D, whereas control cells were exposed to an equivalent percent of DMSO (since actinomycin D was resuspended in DMSO). After 1 hour incubation, IL-1β at 0.25ng/ml was added to all wells (control and treated). RNA was collected at 30, 60, 90, and 180 minutes post-IL-1β and proceeded with PCR (see section 3.5). When examining the effect of α-synuclein on IL-1β-stimulated cells, cells were exposed to desired drug (α-synuclein) and stimulus (IL-1β at 0.25ng/ml) for 2.5 hours, followed by the removal of old media and the addition of fresh serum free media with or without actinomycin D (an inhibitor of transcription). Total RNA was collected at different time points (3, 10, 30, 60, 90, and 180 minutes) to examine mRNA stability. CXCL10 mRNA stablity was measured by quantitative real time PCR (see section 3.5).

### 3.5.5 Statistical analysis of real time RT-PCR results

Following qRT-PCR, quantification of gene expression was evaluated by setting the threshold cycle in the geometric amplification phase on the plot and setting baseline to eliminate any background noise. Relative quantification of gene expression was evaluated by using the comparative computed tomography ($C_T$) method (Hettinger et al., 2001). The expression of target gene was normalized to the expression of endogenous control GAPDH by subtracting the GAPDH $C_T$ value from the corresponding samples target $C_T$ value ($\Delta C_T = C_T$ target-$C_T$ endogenous control). Relative expression was calculated using the comparative $\Delta\Delta C_T$ ($\Delta\Delta C_T$ = mean $\Delta C_T$ of target of treatment group - mean $\Delta C_T$ of target of the calibrator group) method. Fold changes in relative gene expression (RQ) was calculated using $2^{-\Delta\Delta CT}$. The results were expressed as relative expression of target gene for each treatment group in human astroglial cells. If $\Delta C_T$ of treatment group was higher in value compared to $\Delta C_T$ of calibrator group, then RQ was calculated using $2^{-(-\Delta\Delta CT)}$. The value obtained was treated as a negative value, due to less amount of mRNA present in the treatment group (higher $\Delta C_T$) compared to calibrator group (lower $\Delta C_T$).

## 3.6 Analysis of protein expression

### 3.6.1 Bicinchoninic acid (BCA) total protein assay

Levels of total cellular protein content was determined using the bicinchoninic acid (BCA) protein assay as previously described (Davis et al., 2002) in order to normalize data when appropriate. A bovine serum albumin (BSA, Sigma A-7638) standard curve was prepared using BSA, 2.5 M NaOH, and distilled water (see table 4).

**Table 4. Procedure for making BSA standard curve**

| Tube # / BSA concentration | Volume of 2.5 M NaOH | Volume of water | Volume of BSA (2 mg/ml) standard |
|---|---|---|---|
| 1 / 0 µg | 0.1 ml | 0.4 ml | -- |
| 2 / 2 µg | 0.1 ml | 0.35 ml | 0.05 ml |
| 3 / 4 µg | 0.1 ml | 0.3 ml | 0.1 ml |
| 4 / 6 µg | 0.1 ml | 0.25 ml | 0.15 ml |
| 5 / 8 µg | 0.1 ml | 0.2 ml | 0.2 ml |
| 6 / 12 µg | 0.1 ml | 0.1 ml | 0.3 ml |
| 7 / 16 µg | 0.1 ml | -- | 0.4 ml |

Ten µl in duplicate was pipeted from standard curve tubes into wells B1-H2 in a 96-well Falcon plate. Wells A1 and A2 were left empty for blank. Ten µl in duplicate was pipeted from the unknown samples into wells A3-H12 as needed. The volume of BCA working reagent was calculated (1 part 4% cupric sulfate reagent B plus 50 parts BCA reagent A). For each column on the plate, 2 ml of reagent A (Pierce 23223) was prepared. For example, if there were 2 columns of standard and 2 columns of samples, then 4 columns multiplied by 2 ml equaled 8 ml reagent A. To calculate reagent B (Sigma C-2284), divide the total amount of reagent A by 50. For example, 8 ml was divided by 50 to equal 160 µl of reagent B. Reagent B was first added to a clean multi-channel pipet container (plastic, V-shaped rectangle) followed by the addition of reagent A. From this solution, 200 µl was pipetted into each well using a 8-channel pippetor. Plate was incubated at 37.5$^{o}$C for 60 minutes, then read at M570 using a BIO-TEK HT spectrophotometer.

### 3.6.2 Protein isolation

Cellular cytoplasmic and nuclear proteins were isolated using the manufacturer's instructions (Active Motif) and kept on ice throughout the protocol. *Because phospho-IκBα is not highly expressed in A172 cells, duplicate/triplicate wells were pooled together during protein extraction.* Cells were washed with PBS/phosphatase inhibitors (Table 5), removed from plate, and cell suspension was centrifuged for 5 minutes at 500 rpm (4$^{o}$C). The supernatant was discarded and cells were resuspended in 200 µl of 1× hypotonic buffer (Table 5). This resuspension was transferred to pre-chilled microcentrifuged tubes and incubated on ice for 15 minutes. Detergent was added (10 µl/tube) and tubes were vortexed for 10 seconds. Tubes were centrifuged for 30 seconds at 14,000 × g (4$^{o}$C) and the supernatant (protein cytoplasmic fraction) was transferred into pre-chilled tubes and stored at -80$^{o}$C until ready to use. The remaining pellet (protein nuclear fraction) was resuspended in 75 µl Complete Lysis Buffer (Table 5) followed by 10 seconds of high-speed vortexing. The suspension was incubated on ice for 30 minutes on a rocking platform set at 150 rpm. After this, the suspension was vortexed at high-setting for 30 seconds then centrifuged for 10 minutes at 14,000 × g (4$^{o}$C). The resulting supernatant (protein nuclear fraction) was transferred into new pre-chilled tubes and stored at -80$^{o}$C.

**Table 5. Preparation of buffers (per tube)**

| | |
|---|---|
| PBS/Phosphatase Inhibitors | 0.4 ml of 10× PBS + 3.4 ml of distilled water + 0.2 ml of Phosphatase Inhibitors |
| 1× Hypotonic Buffer | 25 µl of 10× Hypotonic Buffer + 225 µl of distilled water |
| Complete Lysis Buffer | 7.5 µl of 10mM DTT + 66.75 µl of Lysis Buffer AM1 + 0.75 µl of Protease inhibitor cocktail |

### 3.6.3 CXCL10 ELISA

A standard dual-antibody solid phase immunoassay (ELISA Development Kit, Peprotech) was used for quantification of secreted CXCL10 in cell culture supernatants, according to manufacturer's instructions.

**Table 6. Preparation of ELISA reagents.**

| | |
|---|---|
| 1× DPBS | 1:10 DPBS [10×] in MilliQ water, Store at 4°C |
| Wash Buffer | 0.05% Tween-20 in 1× DPBS, Prepare 400ml/complete 96-well plate (200 µl Tween in 400ml 1× DPBS), Store at 4°C |
| Block Buffer | 1% BSA in 1× DPBS, Prepare 30ml/complete 96-well plate (300mg BSA in 30ml 1× DPBS), Sterile filter and store at 4°C |
| Standard Diluent | 0.05% Tween-20 and 0.1% BSA in 1× DPBS, Prepare 50ml/complete 96-well plate (25ul Tween + 50mg BSA in 50ml 1× DPBS), Sterile filter and store at 4°C |
| Capture Antibody | Rabbit anti-hIP-10; Reconstituted stock vial in 0.5 ml sterile water for a concentration of 100 µg/ml; resuspended in 1× DPBS |
| Detection Antibody | Biotinylated rabbit-hIP-10; Reconstituted stock vial in 0.25 ml sterile water for a concentration of 100 µg/ml; resuspended in 1× DPBS |
| Avidin Peroxidase-HRP | Resuspended in 12 ml standard diluent |

The appropriate number of ELISA plates (96-well Falcon) were coated and incubated at RT with capture antibody (final concentration of 1 µg/ml, Table 6) the night prior to the rest of the ELISA procedure. The following day, the wells were washed with 200 µl/well wash buffer (Table 6) three times and blotted dry on paper towel. The wells were exposed to 300 µl/well block buffer (Table 6) incubated 1 hour at RT. After incubation with block buffer, wells were washed three times with 300 µl wash buffer. One hundred µl of standards (0-2000 pg/ml) and cell culture supernatants was added to appropriate

antibody-coated wells of the 96-well plate. Liquid contents were aspirated and the wells washed three times with wash buffer (200 µl). Next, 100 µl of antigen-specific biotinylated detection antibody (final concentration of 1 µg/ml, Table 6) was added to each well followed by a 2 hour incubation at room temperature. Wells were aspirated, washed three times (200 µl), and exposed to avidin-peroxidase-horseradish peroxidase (HRP) conjugate (100 µl, Table 6) and incubated for 30 minutes at room temperature. Wells were aspirated and washed three times (200 µl), and 100 µl of ABTS liquid substrate solution (Sigma cat. # A3219) was added to each well followed by 10-15 minute incubation at room temperature. Absorbance was read at 450 nm (with wavelength correction set at 650nm) on the BIO-TEK HT spectrophotometer.

### 3.6.4 Chemiluminescent NF-κB p65 and p50 Transcription Factor assays

The activation of NF-κB was assessed by the nuclear translocation of the NF-κB proteins p65 and p50. The p65 and p50 Transcription Factor kit (Thermo Scientific) was used for quantification of active forms of p65 or p50 from cell protein extracts (see section 3.6.2) according to the manufacturer's instructions. Briefly, 50 µl of working binding buffer (Table 7) was added to appropriate streptavidin-coated wells of a 96-well plate with bound NF-κB biotinylated-consensus sequence. Four µl of wild type or mutant competitor duplex was added, followed by 5 µl of extract, and 2 µl positive control TNFα Activated HeLa cell nuclear extract for 1 hour at room temperature with mild agitation. Liquid contents were aspirated and wells washed three times with 200 µl wash buffer (Table 7). Next, 100 µl of diluted primary antibody (Table 7) was added to each well followed by a 1 hour incubation at room temperature without agitation. Wells were aspirated and washed three times. Diluted secondary antibody (100 µl, Table 7)

were added to each well and incubated for 1 hour at room temperature without agitation. Wells were aspirated, washed four times, 100 µl of chemiluminescent substrate (Table 7) were added to each well and immediately measured (chemiluminescent signal decreases with time). Luminescence was read on the BIO-TEK HT luminometer/spectrophotometer.

**Table 7. Preparation of buffers and antibodies. Volumes per 96-well plate.**

| | |
|---|---|
| Working Binding Buffer | 4.32 ml of Ultrapure Water + 1.152 ml of 5× NFκB Binding Buffer + 288 µl of 20× Poly dIdC |
| Wash Buffer | 45 ml of ultrapure water + 5 ml 10× wash buffer |
| Primary antibody dilution | 12 µl of primary antibody + 12 ml of antibody dilution buffer |
| Secondary antibody dilution | 1 µl of secondary antibody + 10 ml of antibody dilution buffer |
| Chemiluminescent solution | 1:1 combination of Luminol/Enhancer Solution (6 ml) and Stable Peroxide Solution (6 ml), (each strip requires 0.5 ml of each reagent) |

### 3.6.6 Phospho-IκBα (Ser32) Sandwich ELISA assay

Quanitification of the amount of phosphorylated IκBα (Ser32) from cell protein extracts (see section 3.6.2) was measured following manufactorer's instructions (Cell Signaling Technology). Because phosphorylation of IκBα at Ser32/36 is essential for release of active NF-κB, phosphorylation at this site is an excellent marker of NF-κB activation. First, the IκBα capture antibody (100 µl per well at 1:100 in PBS, Table 8) was coated in PBS overnight (17-20 hours) at 4$^{o}$C in 96 well plates. After overnight coating, wells were washed 4 times with wash buffer (200 µl per well, Table 8) and blocked for 2 hours at 37$^{o}$C (150 µl per well, Table 8). Following blocking, wells were washed and 100 µl of cell lysates (see section 3.6.2) was added per well (37$^{o}$C for 2 hours). Wells were washed again and exposed to Phospho-IκBα (Ser32) Detection antibody (100 µl per well at 1:100 in PBS) at 37$^{o}$C for 1 hour. Afterwards, wells were

washed and exposed to secondary anti-rabbit-IgG HRP conjugated antibody (100 µl per well at 1:1000 in PBS) for 30 minutes at 37$^{o}$C. TMB (Cell Signaling #7004), a HRP substrate, was added for color development (100 µl per well) at 37$^{o}$C for 10 minutes. This was followed by the addition of 100 µl of STOP solution (Cell Signaling #7002) per well. Absorbance was read at 450 nm on the BIO-TEK HT spectrophotometer. The absorbance magnitude read is proportional to the quantity of phosphorylated IκBα protein.

**Table 8. Preparation of buffers**

| | |
|---|---|
| Coating Buffer | 1× PBS |
| Wash Buffer | 1× PBS + 0.05% Tween-20 |
| Blocking Buffer | 1× PBS + 0.05% Tween-20 + 1% BSA |

### 3.7 Statistical analysis

GraphPad Prism version 4 (Graph Pad Software Inc, San Deigo, CA) was used for statistical analysis. Results from independent experiments were combined for presentation as the mean + S.E.M. of either actual or percent control values for the number (*n*) of individual samples used for each data point. One-way ANOVA, post-hoc Dunnett's or Neuman-Keuls' multiple comparisons were used for parametric analyses. Differences at $P< 0.05$ were considered significant (* $P<0.05$, ** $P<0.01$, and *** $P<0.001$).

# CHAPTER IV

# RESULTS

## Part I

## Alpha-synuclein modulation of inflammatory signaling in TNFα-stimulated human A172 astroglial cells

### 4.1 Aggregation of α-synuclein alters CXCL10 protein expression in human A172 astroglial cells

Lewy bodies, the pathological hallmark of PD, are mainly composed of fibrillary α-synuclein polymers. However, soluble oligomers of α-synuclein are more toxic to neurons. In order to examine the effects of α-synuclein on inflammatory signaling in human A172 astroglial cells, purified human α-synuclein was 'aged' (aggregated) to obtain an inflammatory-inducing conformation. To decide between a soluble oligomeric formation (7 day aging) and fibrillar polymer (14 day aging), the effect of 7- and 14-day α-synuclein aging on the modulation of CXCL10 protein expression in human A172 astroglial cells was assessed (Figures 8, 10, and 12).

When examining the effects of α-syunclein, acute (24 hour) and chronic (72 hour) exposure of this aggregate was explored in human A172 astroglia (Figure 8A and B). A172 cells were exposed to a range of α-synuclein concentrations (3.7 -300 nM) in the

presence or absence of TNFα (5 ng/ml) (Figures 8-11). Although minimum CXCL10 expression was detected in the unstimulated and α-synuclein alone treatment groups, 24 hour exposure of TNFα alone up-regulated CXCL10 protein expression (Figures 8, 10, and 12). Tumor necrosis factor α-induced CXCL10 production in A172 cells was not significantly affected by α-synuclein co-exposed with TNFα at either acute or chronic exposures of 7-day α-synuclein aggregation (Figure 8A and B). Treatments of 7-day α-synuclein aggregation did not significantly altered astroglial cell viability, as shown in Figures 9A and B.

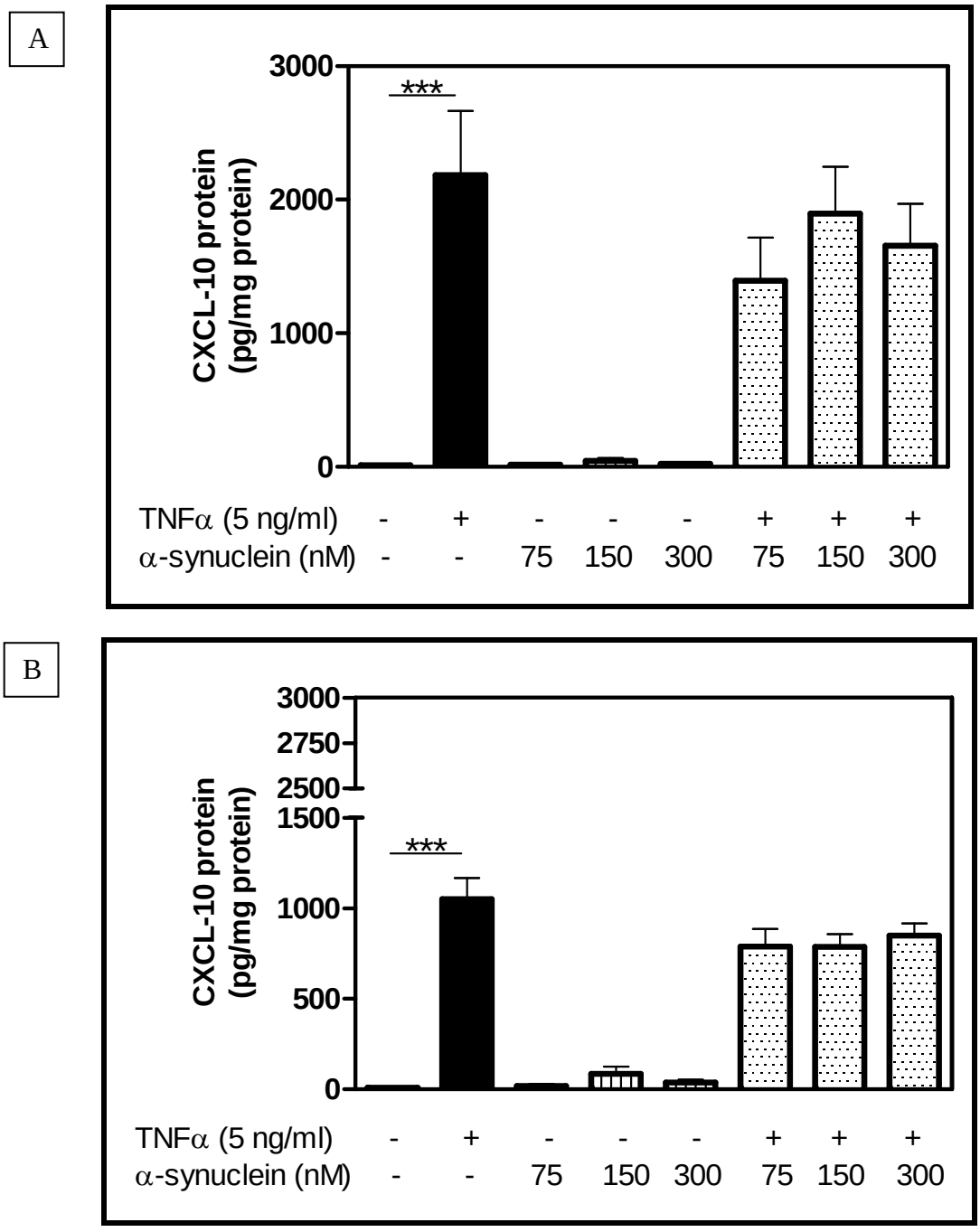


**Figure 8. Effects of acute and chronic exposures of 7day old α-synuclein on TNFα-induced CXCL10 expression in A172 cells.** Cells were exposed to (A) acutely (24h) and (B) chronically (72h) to α-synuclein in the presence or absence of TNFα (5 ng/ml) in serum-free medium for the final 24h. Minimum CXCL10 expression was detected in the unstimulated and α-synuclein alone treatment groups, while 24 hour exposure of TNFα alone up-regulated CXCL10 protein expression. Tumor necrosis factor α-induced CXCL10 production in A172 cells was not significantly affected by α-synuclein co-exposed with TNFα at either acute or chronic exposures of 7-day α-synuclein aggregation. Levels of secreted CXCL10 protein in the media were quantitated by ELISA. Data presented represent the mean + S.E.M. of duplicate measures from 3 independent experiments. Significant differences were determined by one-way ANOVA with Dunnett's post-hoc comparisons relative to TNFα. ***$P < 0.001$ vs TNFα

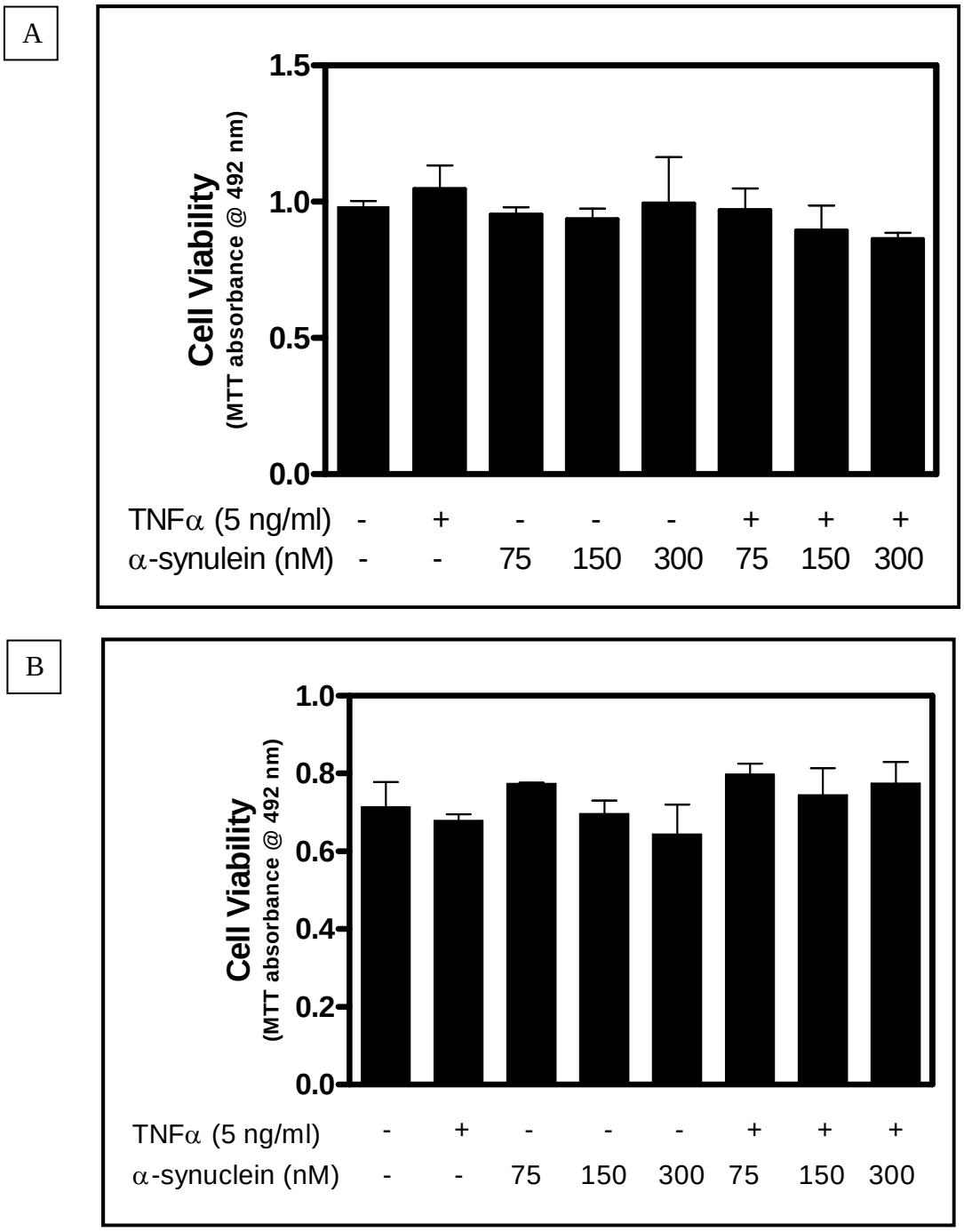


**Figure 9. Effect of 7-day old α-synuclein and TNFα on cytotoxicity in human astroglial cells cytotoxicity.** Cells were exposed to (A) acutely (24h) and (B) chronically (72h) to α-synuclein in the presence or absence of TNFα (5 ng/ml) in serum-free medium for 24h. As shown in (A) and (B), A172 cells treated with 7-day α-synuclein aggregation did not significantly alter astroglial cell viability. Cell viability was assessed using the MTT assay. Data presented represent the mean + S.E.M. of 3 measures from a single independent experiment. No significant differences ($P$>0.05) among treatment were determined by one-way ANOVA.

The effects of a 14-day aging of α-syunclein resulted in a different trend (Figure 10A and B). Although acute and chronic exposure of α-synuclein to A172 cells produced minimal CXCL10 protein levels in the absence of TNFα, α-syunclein co-exposed with TNFα (final 24 hours) significantly ($P<0.05$) enhanced TNFα-induced CXCL10 production in human astroglial cells. The up-regulation was detected following an acute exposure of 11 nM α-synuclein; yet, this effect was lost as the concentration of α-syunclein increased (33-300 nM) (Figure 10A). Chronic exposure to 75 and 300 nM α-syunclein also resulted in an enhancement of CXCL10 expression relative to TNFα treated cells (Figure 10B). The modulation did not appear to be due to cell viability variations among treatments (Figure 11). Examination of TNFα at lower concentrations (0.1-2.5 ng/ml) dose-dependently increased the expression of CXCL10 in A172 cells; however, the effect of α-syunclein (3.7-33 nM) at the lower TNFα concentrations did not enhance the TNFα-induced CXCL10 protein expression (Figure 12A and B). Furthermore, data suggest that TNFα at the concentration of 5 ng/ml is required to provide a threshold level needed for α-syunclein to have an effect.

Overall, 14-day aged α-synuclein resulted in significant modulation of the production of TNFα-induced CXCL10 protein. Furthermore, acute exposure of α-syunclein resulted in a concentration-dependent production of CXCL10 protein. Consequently, all subsequent experiments were performed with 24 hour administration of α-synuclein aged for 14 days.

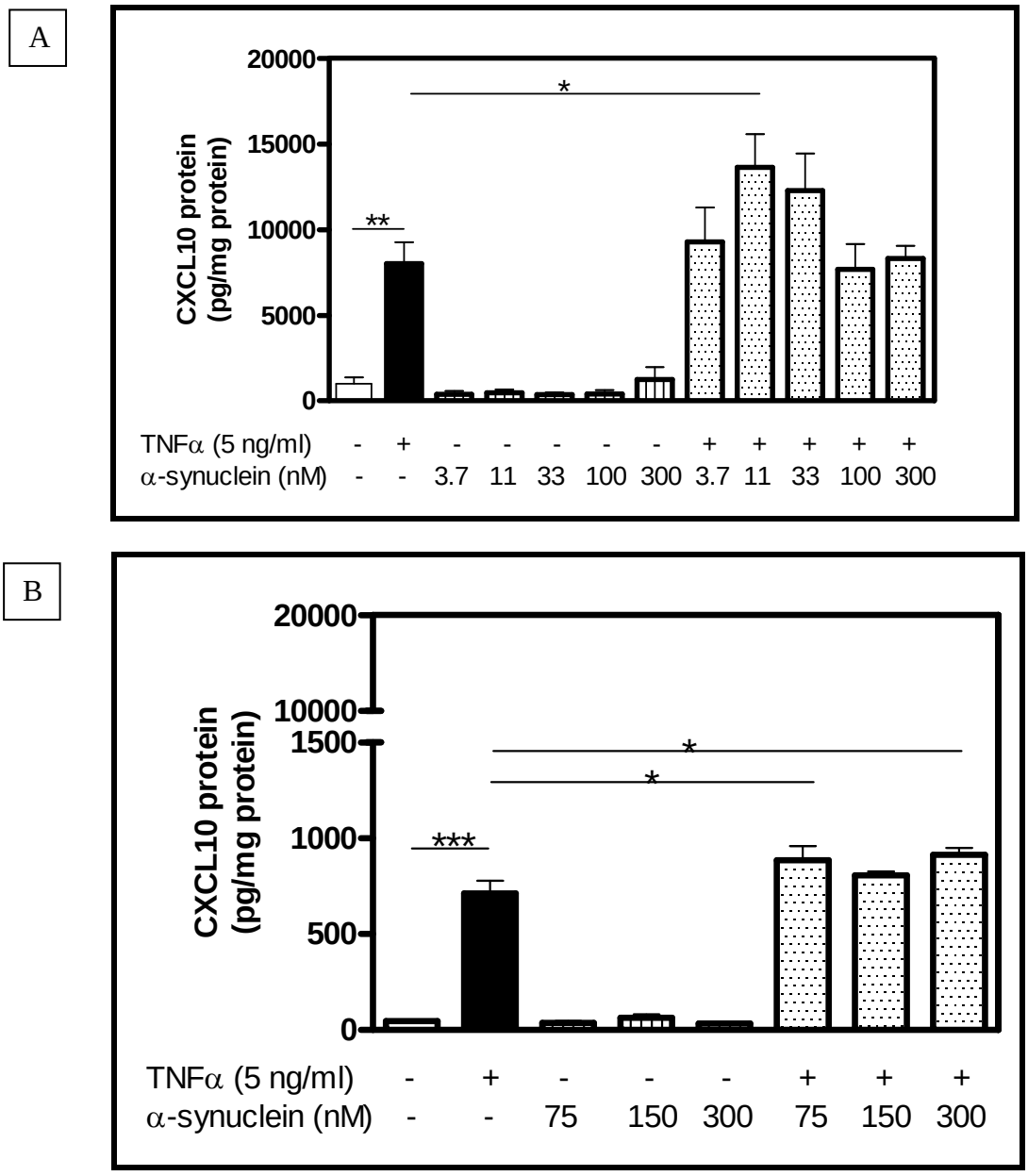


**Figure 10. Effects of acute and chronic exposures of 14-day old α-synuclein on TNFα-induced CXCL10 expression in A172 cells.** Cells were exposed to (A) acutely (24h) and (B) chronically (72h) to α-synuclein in the presence or absence of TNFα (5 ng/ml) in serum-free medium for 24 h. Although the addition of α-synuclein to A172 cells produced minimal CXCL10 protein levels in the absence of TNFα (A and B), α-syunclein co-exposed with TNFα (final 24 hours) significantly enhanced TNFα-induced CXCL10 production. The up-regulation was detected following an acute exposure of 11 nM α-synuclein (A), while chronic exposure of 75 and 300 nM α-syunclein also resulted in an enhancement of CXCL10 expression relative to TNFα treated cells (B). Levels of secreted CXCL10 protein in the media were quantitated by ELISA. Data presented represent the mean + S.E.M. of 2-3 measures from 1-7 independent experiments. Significant differences were determined by one-way ANOVA with Dunnett's post-hoc comparisons relative to TNFα. **P* < 0.05 vs TNFα, **P* < 0.01 vs TNFα , *** *P* < 0.001 vs TNFα.

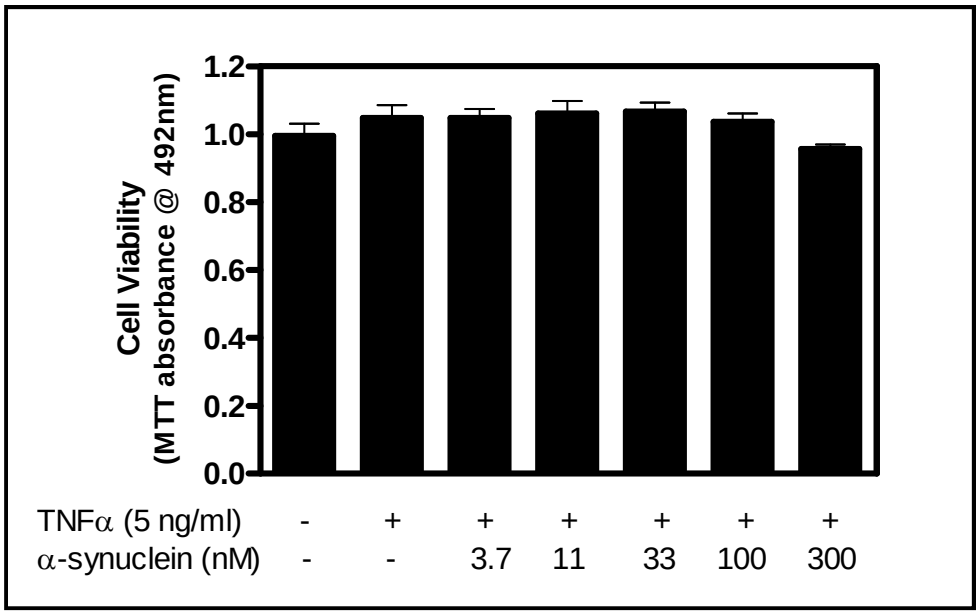


**Figure 11. Effect of 14-day old α-synuclein and TNFα on cytotoxicity in human astroglial cells cytotoxicity.** Cells were exposed to TNFα (5 ng/ml) in the presence or absence of acute (24h) exposure of α-synuclein in serum-free medium for 24 h. Untreated A172 cells and cells treated with TNFα +/- α-synuclein did not result in differing cell viability levels. Cell viability was assessed using the MTT assay. Data presented represent the mean + S.E.M. of duplicate measures from 5 independent experiments. No significant differences ($P$>0.05) among treatment were determined by one-way ANOVA.

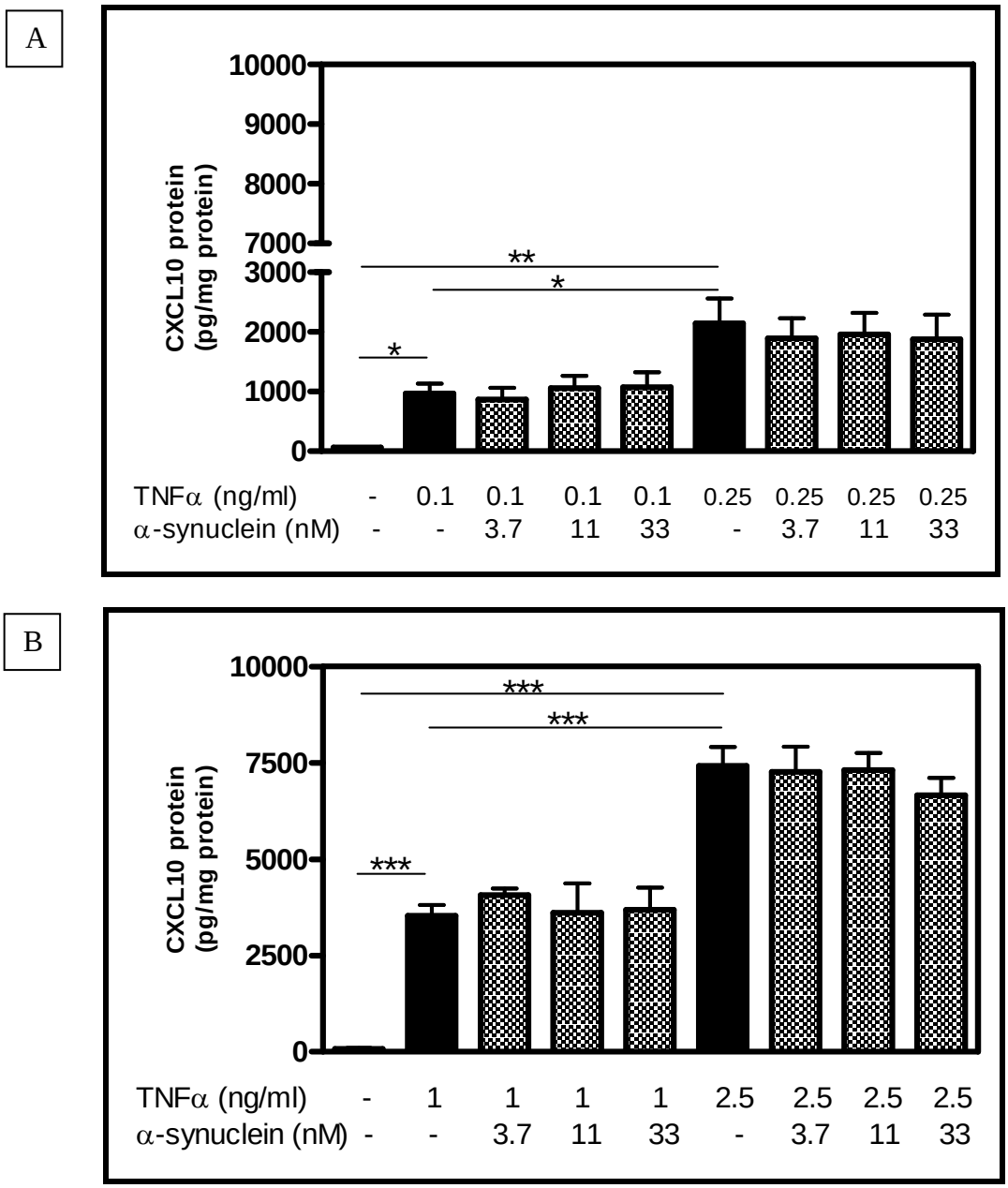


**Figure 12. Effects of 14-day old α-synuclein on TNFα-induced CXCL10 expression in A172 cells.** Cells were exposed to (A) TNFα (0.1 and 0.25 ng/ml) (B) TNFα (1 and 2.5 ng/ml) in the presence or absence of α-synuclein (3.7-33 nM) in serum-free medium for 24h. Examination of TNFα at lower concentrations (0.1-2.5 ng/ml) dose-dependently increased the expression of CXCL10 in A172 cells; however, the effect of α-syunclein (3.7-33 nM) did not enhance the TNFα-induced CXCL10 protein expression (A and B). Levels of secreted CXCL10 protein in the media were quantitated by ELISA. Data presented represent the mean + S.E.M. of duplicate measures from 3 independent experiments. Significant differences were determined by one-way ANOVA with Dunnett's post-hoc comparisons relative to TNFα. *$P < 0.05$ vs TNFα, **$P < 0.01$ vs TNFα , *** $P < 0.001$ vs TNFα

## 4.2 Increased NF-κB signaling by α-synuclein in TNFα-stimulated human A172 astroglial cells

The activation of NF-κB was assessed by the nuclear translocation of the NF-κB proteins p65 and p50. CXCL10 expression is regulated in part at the transcriptional level by the transcription factor NF-κB. Levels of active nuclear NF-κB protein p65 were evaluated to determine relative NF-κB modulations by α-synuclein. To determine if the 14-day aged human α-synuclein has an effect on NF-κB, astroglial cells were exposed to the α-synuclein and TNFα treatment that significantly induced protein expression (Figure 10A). As shown in Figure 13, unstimulated and 11 nM α-synuclein alone (24 hour) resulted in a minimal activation of NF-κB; however, TNFα (5 ng/ml) increased the activation as early as 10 minutes post-TNFα exposure. The enhancement of NF-κB activation by TNFα was remained elevated (30 minutes), but co-exposure to α-synuclein did not significantly alter this modulation by TNFα (Figure 13).

Upon further examination using additional α-synuclein concentrations (3.7 and 33 nM), it was determined that NF-κB activation was significantly up-regulated by α-synuclein relative to TNFα treated cells (Figure 14A). Specifically, aggregated α-synuclein at 3.7 and 33 nM significantly ($P<0.001$ and $P<0.01$, respectively) induced NF-κB activation at 3 minutes (Figure 14A). However, this effect by α-synuclein was lost at subsequent time points of 10 and 30 minutes (Figure 14B and C). Nuclear factor – κB activation was increased in TNFα treated cells, yet showed minimum induction in the presence of α-synuclein alone and unstimulated cells (Figure 13 and 14).

Therefore, 11 nM of α-synuclein did not increase NF-κB activation relative to TNFα alone, yet the enhancement of NF-κB activation at the level of p65-DNA binding was detected by other α-synuclein concentrations (3.7 and 33 nM) in A172 cells.

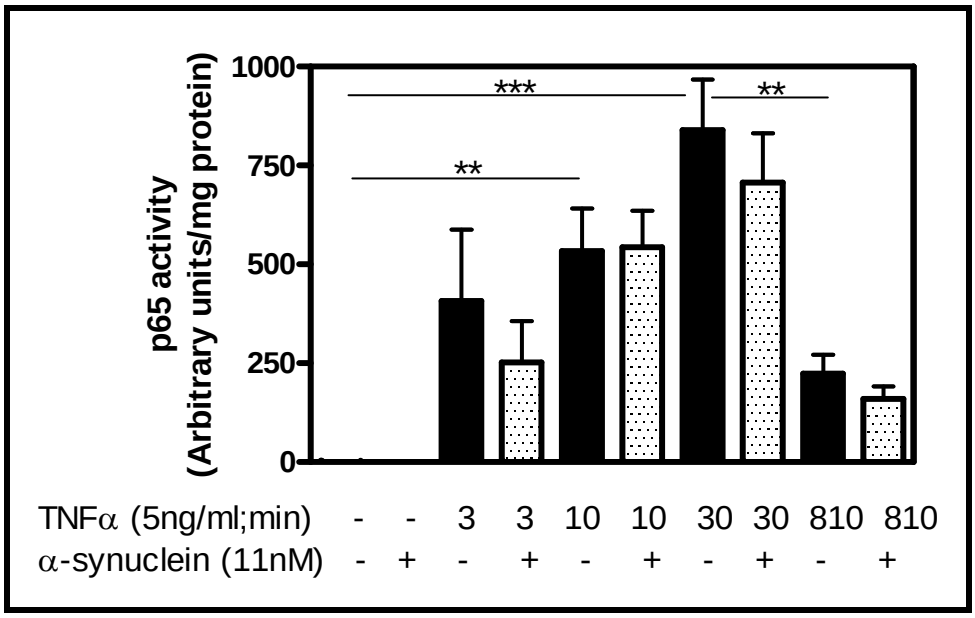


**Figure 13. Alpha-synuclein did not enhance inflammatory-induced active NF-κB signaling protein in human A172 astroglial cells.** Cells were exposed to α-synuclein in serum-free medium for 24 h. Also, during the last 3, 10, 30, and 810 min, cells were co-exposed to TNFα (5 ng/ml). Unstimulated cells and cells exposed to 11 nM α-synuclein alone resulted in a minimal activation of NF-κB; however, TNFα increased the activation as early as 10 minutes post-TNFα exposure. The enhancement of NF-κB activation by TNFα was remained elevated (30 minutes), but co-exposure to α-synuclein did not significantly alter this modulation by TNFα. Arbitrary binding activity of NF-κB p65 signaling protein was determined chemiluminescently using nuclear extracts. Data presented represent the mean + S.E.M. of duplicate measures from 2 independent experiments. Significant differences were determined by one-way ANOVA with Dunnett's post-hoc comparisons relative to TNFα. $^{**}P < 0.01$ vs TNFα , $^{***}P < 0.001$ vs TNFα

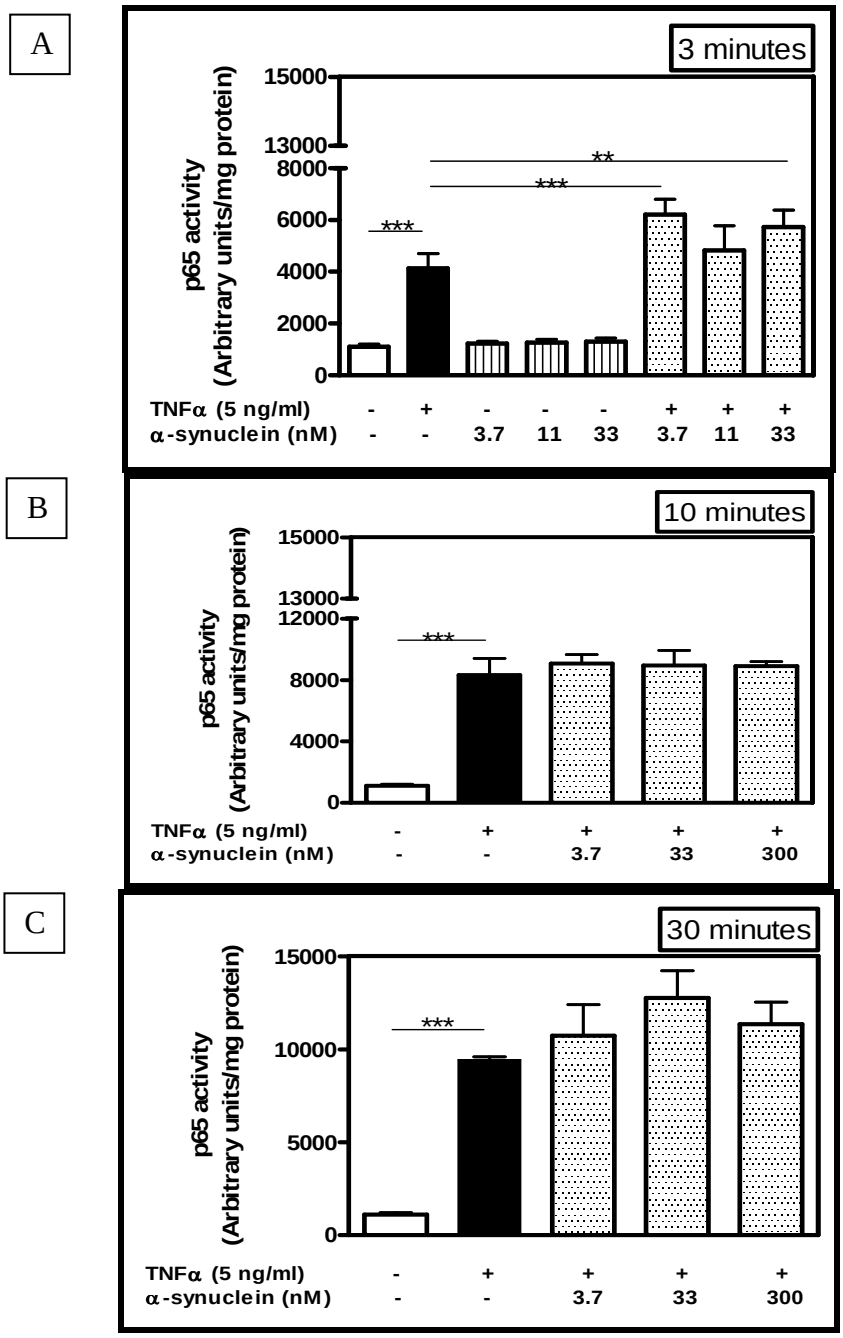


**Figure 14. Lower α-synuclein concentration potentiates inflammatory-induced active NF-κB signaling protein in human A172 astroglial cells.** Cells were exposed to α-synuclein in serum-free medium for 24 h. Also, during the last 3, 10 and 30min, cells were co-exposed to TNFα (5 ng/ml). NF–κB activation was increased in TNFα treated cells, yet showed minimum induction in the presence of α-synuclein alone and unstimulated cells (A). At 3 minutes, aggregated α-synuclein at 3.7 and 33 nM in the presence of TNFα significantly ($P$<0.001 and $P$<0.01, respectively) induced NF-κB activation (A) relative to TNFα alone. However, this effect by α-synuclein was lost at subsequent time points of 10 and 30 minutes (B and C). Arbitrary binding activity of NF-κB p65 signaling protein was determined chemiluminescently using nuclear extracts. Data presented represent the mean + S.E.M. of 2-3 measures from 1-4 independent experiments. Significant differences were determined by one-way ANOVA with Dunnett's post-hoc comparisons relative to TNFα. ** $P$ < 0.01 vs TNFα, *** $P$ < 0.001 vs TNFα.

## 4.3 Supplementary Data: Elevated nitrite production by α-synuclein in cytokine-stimulated human A172 astroglial cells

To determine the effects of α-synuclein on iNOS induction, nitrite accumulation from treatment media was used as an indicator of inflammatory enzyme activity. Stimulation of A172 cells by a cytokine mixture (TNFα + IL-1β + IFNγ) for 24 hours resulted in a significant induction of nitrite levels relative to unstimulated cells. This induction was not affected by the addition of α-synuclein (24 hour exposure) at concentrations ≤100 nM, as indicated by no significant change in nitrite production relative to cytokine mixture (Figure 15). Conversely, the presence of 300 nM significantly ($P<0.05$) potentiated the amount of nitrite production induced by the cytokine mixture. As shown in Figure 15, unstimulated cells only produced minimal nitrite.

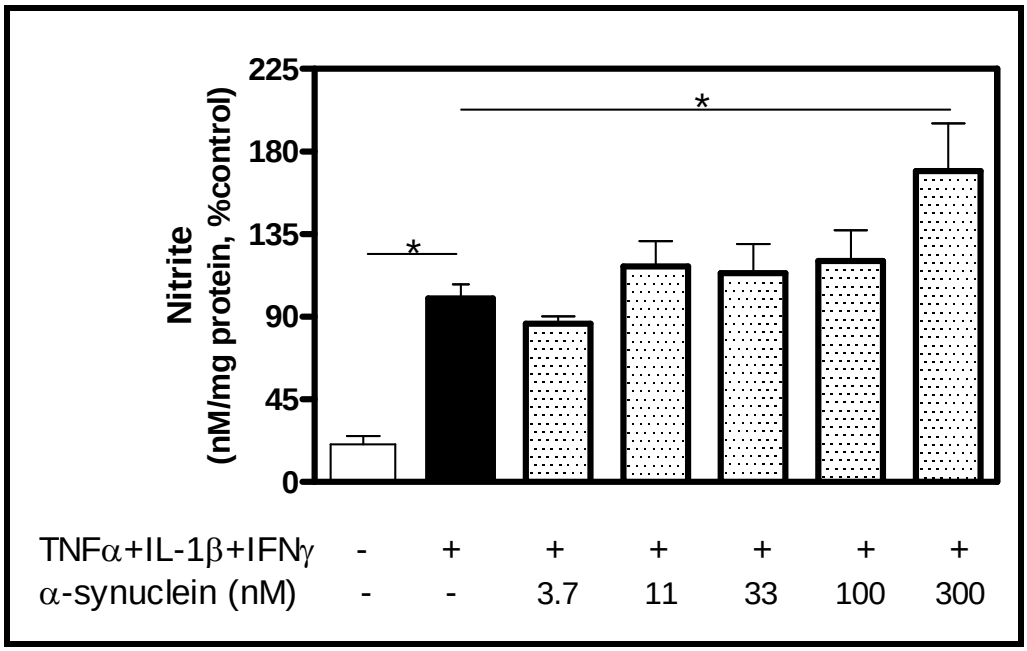


**Figure 15. Alpha-synuclein enhanced inflammatory-induced nitrite production in human A172 astroglial cells.** Cells were exposed to α-synuclein and co-exposed to TNFα (30 ng/ml), IL-1β (5 ng/ml), and IFNγ (100 ng/ml) for 24h. A172 cells stimulated by the cytokine mixture resulted in a significant induction of nitrite levels relative to unstimulated cells. This induction was not affected by the addition of α-synuclein (24 hour exposure) at concentrations ≤100 nM; however, α-synuclein at 300 nM significantly ($P$<0.05) potentiated the amount of nitrite production induced by the cytokine mixture. Nitrite accumulation in the culture media was determined spectrophotometrically using the Griess reagent. Data were normalized as percent control within each independent experiment and represent the mean + S.E.M. of 1-2 measures from 2 independent experiments. Significant differences were determined by one-way ANOVA with Dunnett's post-hoc comparisons. *$P$ < 0.05 vs control.

## Part II

## Alpha-synuclein modulation of inflammatory signaling in IL-1β-stimulated human A172 astroglial cells

### 4.4 Enhancement of CXCL10 protein expression by α-synuclein on IL-1β-induced CXCL10 expression in human A172 astroglial cells

To assess α-synuclein's effect on the production of CXCL10 induced from another critical PD inflammatory mediator, IL-1β-stimulated astrocytes were exposed to concentrations of α-synuclein (3.7-33 nM) and analyzed relative to IL-1β alone. Under unstimulated conditions, CXCL10 protein production was constitutively expressed at very low levels (Figure 16). Minimum expression of this chemokine was demonstrated by α-synuclein alone treatments (24 hour exposure, Figure 10A). When astroglial cells were exposed to IL-1β for 24 hours, results demonstrated an enhanced chemokine production at $\geq$ 0.25 ng/ml relative to unstimulated cells. When cells were co-exposed to both α-synuclein and IL-1β for 24 hours, α-synuclein at 33 nM significantly potentiated IL-1β (0.25 ng/ml)-induced CXCL10 expression. The up-regulation was not due to variations in cell viability among treatments (Figure 17). However, enhancement of CXCL10 protein by α-synuclein was not continued with higher IL-1β concentrations (1 and 5 ng/ml, Figure 16B). Therefore, the data suggest that α-synuclein significantly up-regulated IL-1β-induced CXCL10 production, however, this effect by α-synuclein was lost as the concentration of IL-1β increased.

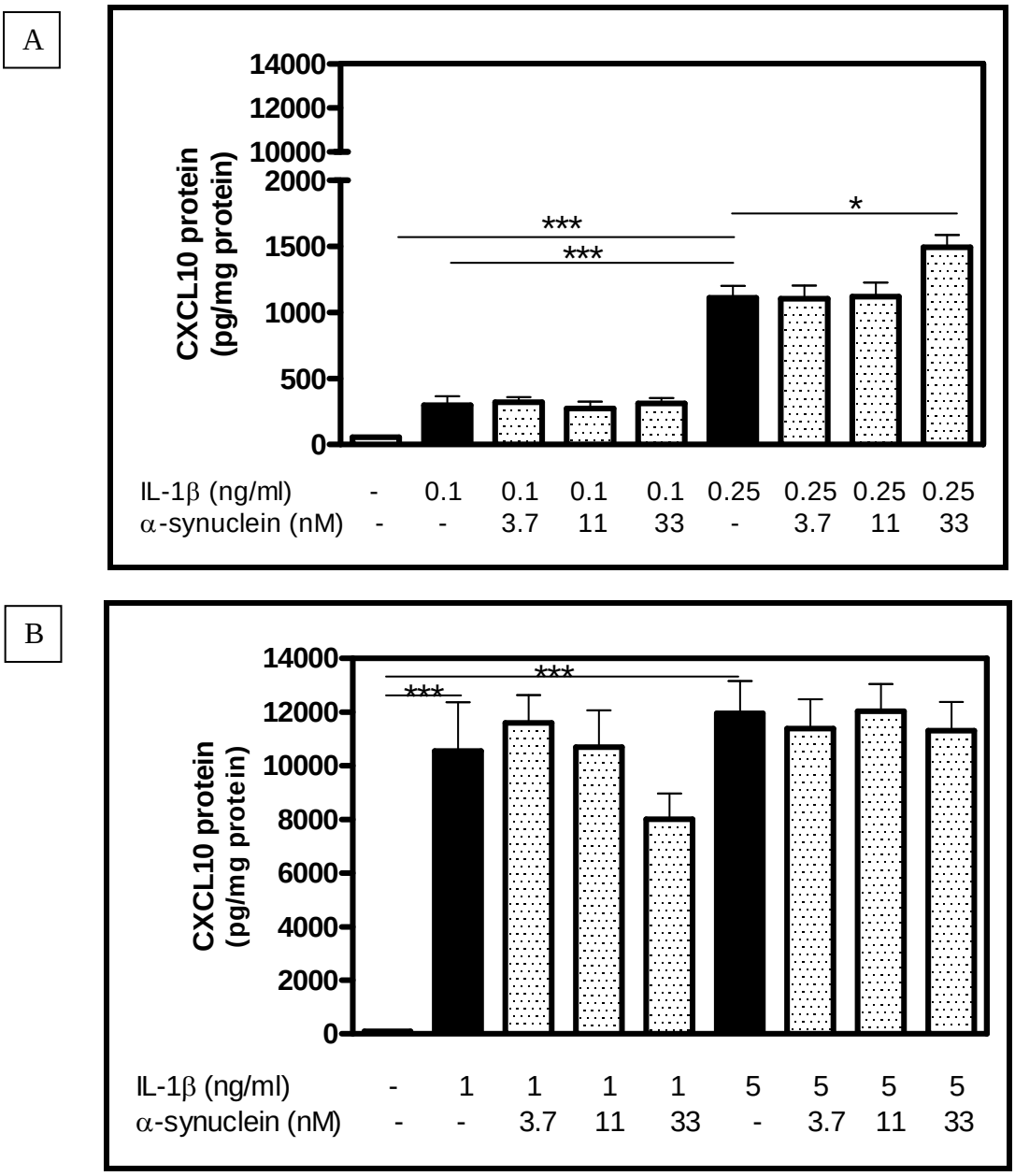


**Figure 16. Effects of α-synuclein on IL-1β-induced CXCL10 expression in A172 cells.** Cells were exposed to IL-1β at (A) 0.1 and 0.25 ng/ml and (B) 1 and 2.5 ng/ml in the presence or absence of α-synuclein (3.7-33 nM) in serum-free medium for 24 h. Under unstimulated and α-synuclein alone conditions, minimum expression of CXCL10 protein was expressed (A). Astroglial cells were exposed to IL-1β resulted in an enhanced chemokine production at ≥ 0.25 ng/ml relative to unstimulated cells. Alpha-synuclein at 33 nM significantly potentiated IL-1β (0.25 ng/ml)-induced CXCL10 expression in cells co-exposed to both α-synuclein and IL-1β. This up-regulation by α-synuclein was not seen continued with higher IL-1β concentrations (B). Levels of secreted CXCL10 protein in the media were quantitated by ELISA. Data presented represent the mean + S.E.M. of duplicate measures from 3 independent experiments. Significant differences were determined by one-way ANOVA with Dunnett's post-hoc comparisons. *$P < 0.05$ vs IL-1β, ***$P < 0.05$ vs IL-1β.

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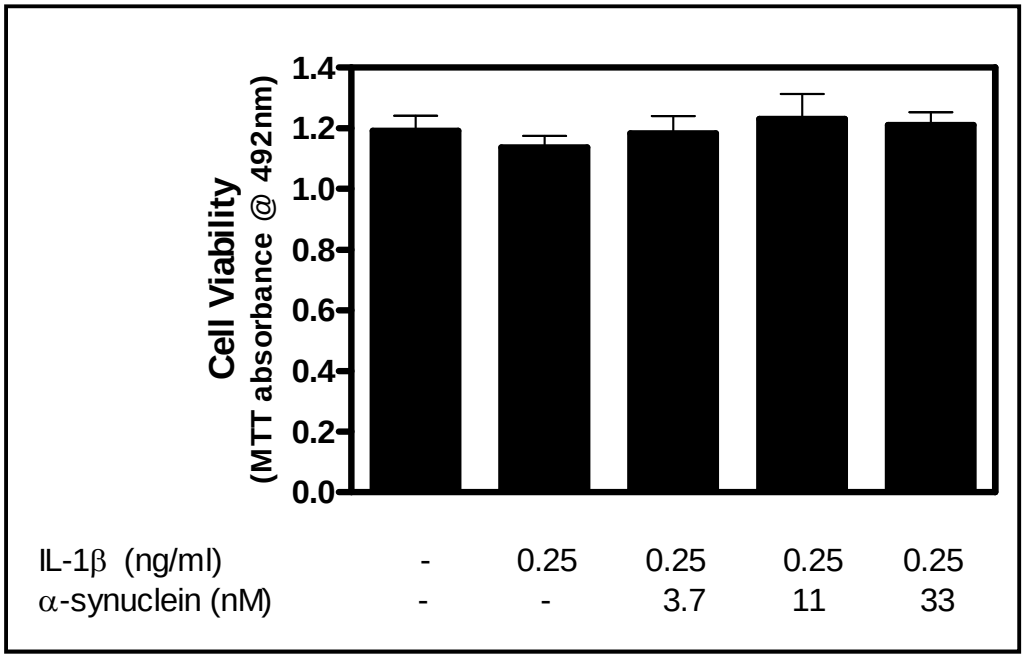



**Figure 17. Effect of α-synuclein and IL-1β on cytotoxicity in human astroglial cells cytotoxicity.** Cells were exposed to IL-1β (0.25 ng/ml) in the presence or absence of α-synuclein (3.7-33 nM) in serum-free medium for 24 h. Untreated A172 cells and cells treated with IL-1β +/- α-synuclein did not result in differing cell viability levels. Cell viability was assessed using the MTT assay. Data presented represent the mean + S.E.M. of triplicate measures from 2 independent experiments. No significant differences ($P$>0.05) among treatment were determined by one-way ANOVA.

## 4.5 Alpha-synuclein did not modulate IL-1β-induced CXCL10 mRNA expression in human A172 cells

To identify the role of α-synuclein on CXCL10 mRNA expression, astroglial cells were exposed to IL-1β and α-synuclein over several time periods. The experiments used concentrations of IL-1β and α-synuclein that induced significant CXCL10 protein expression (Figure 16A) and were analyzed relative to CXCL10 mRNA expression levels observed by IL-1β at the 2-hour time point. Initially identifying the effects of IL-1β alone on chemokine mRNA, results demonstrated a time-dependent effect of this cytokine at 0.25 ng/ml on CXCL10 mRNA expression (Figure 18A). As illustrated in Figure 18A, IL-1β potentiated the expression of CXCL10 mRNA in astroglia and peaked at 8 hours post-exposure. CXCL10 mRNA expression produced by IL-1β at later time points (≥16 hours) returned to levels similar to 2 hour post-treatment. When cells were co-exposed with 33 nM α-synulcein and IL-1β, significant alterations of inflammatory-induced chemokine mRNA expression by α-synuclein were not detected (Figure 18B). Furthermore, other α-synulcein concentrations (3.7 and 11 nM) did not significantly alter IL-1β-induced CXCL10 mRNA expression (Figure 18B).

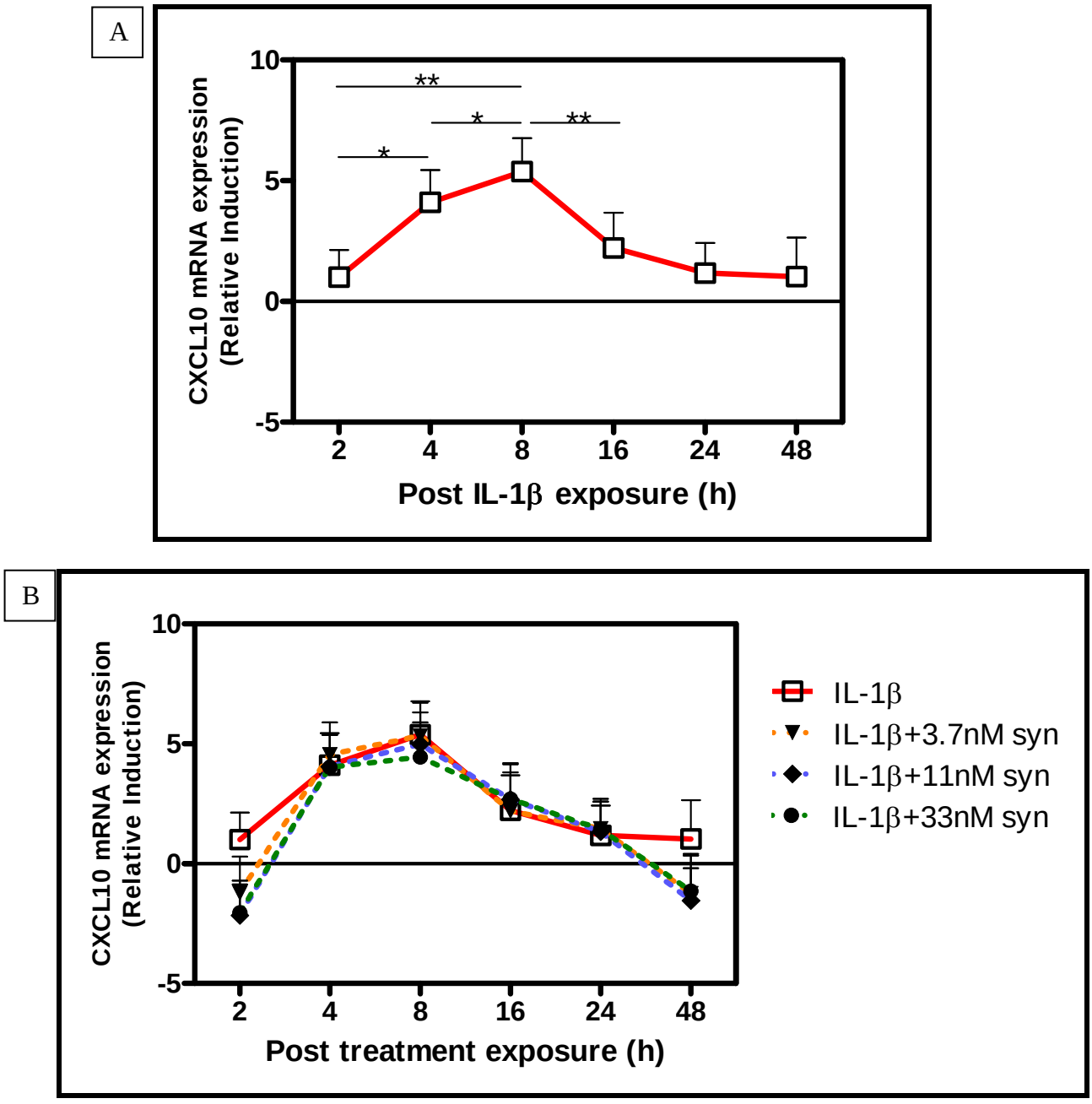
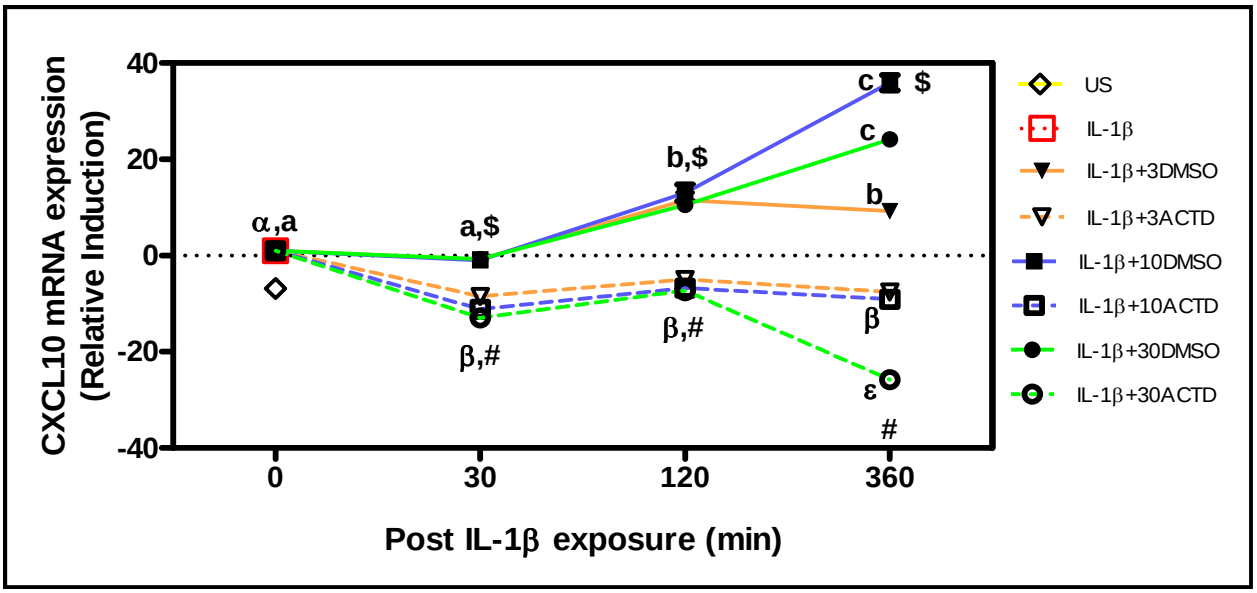



**Figure 18. Effect of α-synuclein on IL-1β-stimulated CXCL10 mRNA expression in human astroglial cells.** Cells were exposed to (A) IL-1β (0.25 ng/ml) alone or (B) in the presence of α-synuclein (3.7-33 nM) in serum-free medium for indicated hours. CXCL10 mRNA levels were potentiated by IL-1β in astroglia with a peak at 8 hours post-exposure (A). The addition of α-synuclein (at all concentrations) to IL-1β-stimulated cells did not significantly alter IL-1β-induced CXCL10 mRNA expression (B). CXCL10 and GAPDH mRNA was assessed using quantitative real time RT-PCR. Data were normalized to GAPDH within each independent experiment and expressed as relative induction of IL-1β at 2 h post-treatment. Data presented represent the mean + S.E.M. of duplicate measures from 4-8 independent experiments. Significant differences among treatment were determined by one-way ANOVA with Dunnett's post-hoc comparisons. $*P < 0.05$ vs IL-1β, $**P < 0.01$ vs IL-1β.

## 4.6 Alpha-synuclein mediates the stability of IL-1β-induced CXCL10 mRNA in human A172 cells

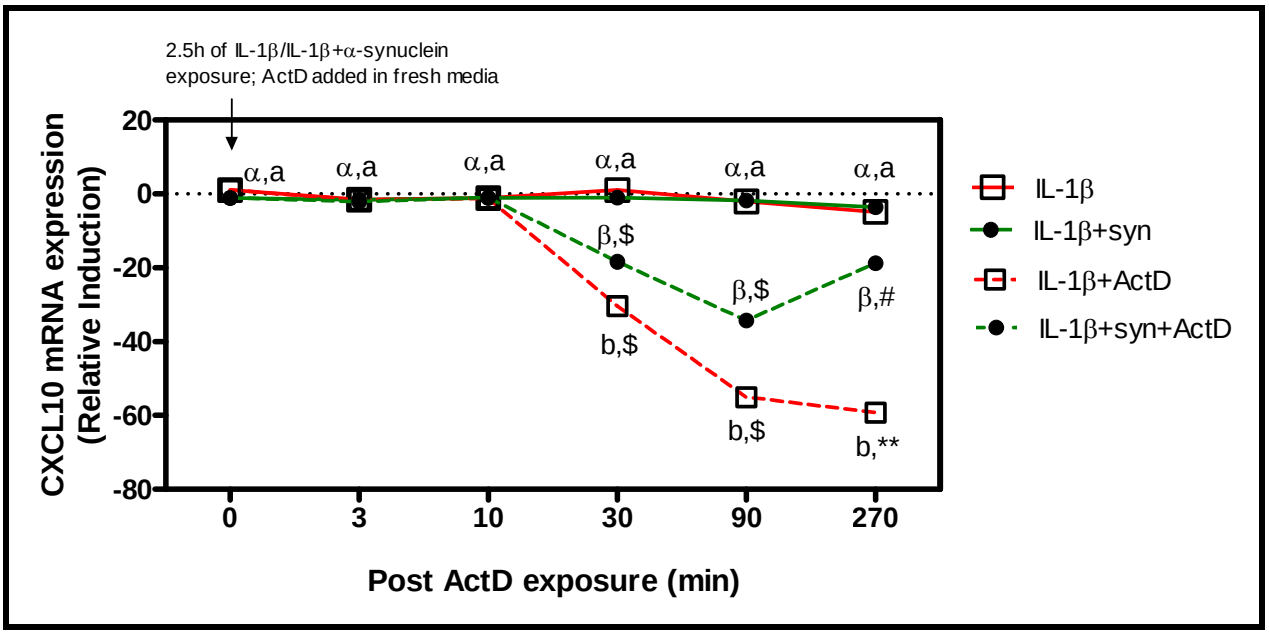
CXCL10 protein expression was increased in the presence of α-synuclein, whereas the mRNA accumulation was not affected by this protein. Therefore, the induction of CXCL10 protein by α-synuclein may be mediated, in part, by an increase in mRNA stability. To determine whether α-synuclein mediates CXCL10 mRNA stability, A172 cells were exposed to the transcription inhibitor actinomycin D at varying times to inhibit *de novo* mRNA synthesis. It was first necessary to verify the effectiveness of actinomycin D in human A172 cells prior to initiating α-synuclein experiments. Human A172 cells were pre-treated with either actinomycin D (3, 10, 30 µg/ml) or control vehicle (3, 10, 30 µg/ml DMSO) 1 hour prior to the addition of IL-1β (0.25 ng/ml) (Figure 19). Data was analyzed relative to CXCL10 mRNA expression levels observed by the IL-1β-only treatment that ran concurrently with the pretreatments. Treatment groups of cells exposed to IL-1β in the presence of control vehicle showed a gradual increase of CXCL10 mRNA, with significant mRNA levels detected at 120 minutes post-treatment (Figure 19). A172 cells pre-treated with actinomycin D inhibited the synthesis of CXCL10 mRNA transcription relative to IL-1β with DMSO treatments, and significant inhibition by actinomycin D was observed at earliest time point (30 minutes) and across time. Consequently, the transcription of *de novo* CXCL10 mRNA transcripts in A172 cells is effectively inhibited by actinomycin D, as demonstrated in Figure 19.




**a, b,c** represent statistical analysis within treatment group IL-1β + DMSO (3,10,30 µg/ml) across time
**α, β, ε** represent statistical analysis within treatment group IL-1β + ActD (3,10,30 µg/ml) across time
**$, #** represent statistical ananlysis between treatment groups IL-1β + DMSO (3,10,30 µg/ml) and IL-1β + ActD (3,10,30 µg/ml) within each time point

**Figure 19. Inhibition of *de novo* CXCL10 mRNA transcription by actinomycin D in IL-1β-stimulated human astroglial cells.** Cells were pretreated with DMSO or ActD (3,10,30 µg/ml) in serum-free medium one hour prior to the addition of IL-1β (0.25 ng/ml) for the indicated times. Concurrent to 1 h pretreatment, IL-1β alone treatment also ran for one hour. Cells exposed to IL-1β in the presence of control vehicle showed a gradual increase of CXCL10 mRNA, with significant mRNA levels detected at 120 minutes post-treatment. A172 cells pre-treated with actinomycin D inhibited the synthesis of CXCL10 mRNA transcription relative to IL-1β with DMSO treatments, with significant inhibition by actinomycin D at the earliest time point (30 minutes) and across time. CXCL10 and GAPDH mRNA was assessed using quantitative real time RT-PCR. Data were normalized to GAPDH within each independent experiment and expressed as relative induction of IL-1β alone (1 h treatment). Data presented represent the mean + S.E.M. of duplicate measures from 2 independent experiments. Lines and time points with different superscript letters/symbols are significantly ($P < 0.05$) different as determined by one-way ANOVA with Newman-Keul's post-hoc comparisons.

Next, actinomycin D was used to examine possible post-transcriptional effects of α-synuclein on CXCL10 mRNA stability (Figure 20). CXCL10 mRNA production was induced by a 2.5 hour pretreatment of either IL-1β (0.25 ng/ml) or α-synuclein (33 nM) with IL-1β in order to produce adequate mRNA production. Because the use of 10 µg/ml concentration of actinomycin D on astroglia was consistant with others (Gardner et al., 2006; Kasahara et al., 1991; Yao et al., 2005), this concentration was used to examine α-synuclein's role on stability. After the pretreatment, media was replaced with fresh serum-free media in the presence or absence of actinomycin D (10 µg/ml). A172 cells were exposed to actinomycin D for various time points (3 – 270 minutes) and the amount of CXCL10 mRNA was determined and relatively quanified to IL-1β at the 0 time point (Figure 20). Results showed no difference of CXCL10 mRNA levels between IL-1β and IL-1β with α-synuclein in the absence of actinomycin D. Conversely, the addition of actinomycin D to treatments resulted in a time-dependent decrease in CXCL10 mRNA with a significant mRNA decay detected by 30 minutes. However, less decay was seen over time when α-synuclein was present. Specifically, significantly less mRNA decay was detected in the α-synuclein+IL-1β treatment relative to IL-1β at 270 minutes post-actinomycin D treatment (Figure 20). Overall, the observations reveal that α-synuclein exerts post-transcriptional effects which contribute to increased CXCL10 mRNA stability in human A172 cells (Figure 20).




**a, b** represent statistical analysis within and between treatment groups IL-1β and IL-1β + 10 µg/ml ActD across time

**α, β** represent statistical analysis within and between treatment groups IL-1β + 33nM syn and IL-1β + 33nM syn + 10 µg/ml ActD across time

**$, #, **** represent statistical analysis between treatment groups IL-1β + 10 µg/ml ActD and IL-1β + 33nM syn + 10 µg/ml ActD across time

**Figure 20. Effect of α-synuclein on CXCL10 mRNA stability in IL-1β-stimulated human astroglial cells.** Cells were pretreated with IL-1β (0.25 ng/ml) in the presence or absence of α-synuclein (33 nM) for 2.5 h. Actinomycin D or DMSO (3,10,30 µg/ml) was exposure to cells in fresh serum-free medium for the indicated times. CXCL10 mRNA levels between IL-1β and IL-1β with α-synuclein in the absence of actinomycin D showed no difference. Conversely, the addition of actinomycin D to treatments resulted in a time-dependent decrease in CXCL10 mRNA with a significant mRNA decay detected by 30 minutes. Less decay was seen over time when α-synuclein was present compared to IL-1β with actinomycin D alone, specifically at 270 minutes post-actinomycin D treatment. CXCL10 and GAPDH mRNA was assessed using quantitative real time RT-PCR. Data were normalized to GAPDH within each independent experiment and expressed as relative induction of IL-1β at 0 time point. Data presented represent the mean + S.E.M. of duplicate measures from 2 independent experiments. Lines and time points with different superscript letters/symbols are significantly ($P < 0.05$) different as determined by two-way and one-way ANOVA with Newman-Keul's post-hoc comparisons.

## 4.7 Alpha-synuclein does not affect NF-κB signaling in IL-1β-stimulated human A172 astroglial cells

### 4.7.1 Levels of I-κBα phosphorylation were not enhanced by α-synuclein

CXCL10 expression is regulated in part at the transcriptional level by the transcription factor NF-κB. Levels of phosphorylated I-κBα were evaluated to determine relative NF-κB modulations by α-synuclein (Figure 21). A172 astroglial cells were exposed to the α-synuclein and IL-1β treatment that significantly induced CXCL10 protein expression (Figure 16A). The presence of IL-1β resulted in no significant enhancement in the levels of phosphorylated I-κBα in A172 cells relative to unstimulated cells. Moreover, astroglial cells co-exposed with both IL-1β and α-synuclein did not result in a significant modulation of phosphorylated I-κBα levels relative to IL-1β-induced levels (Figure 21).

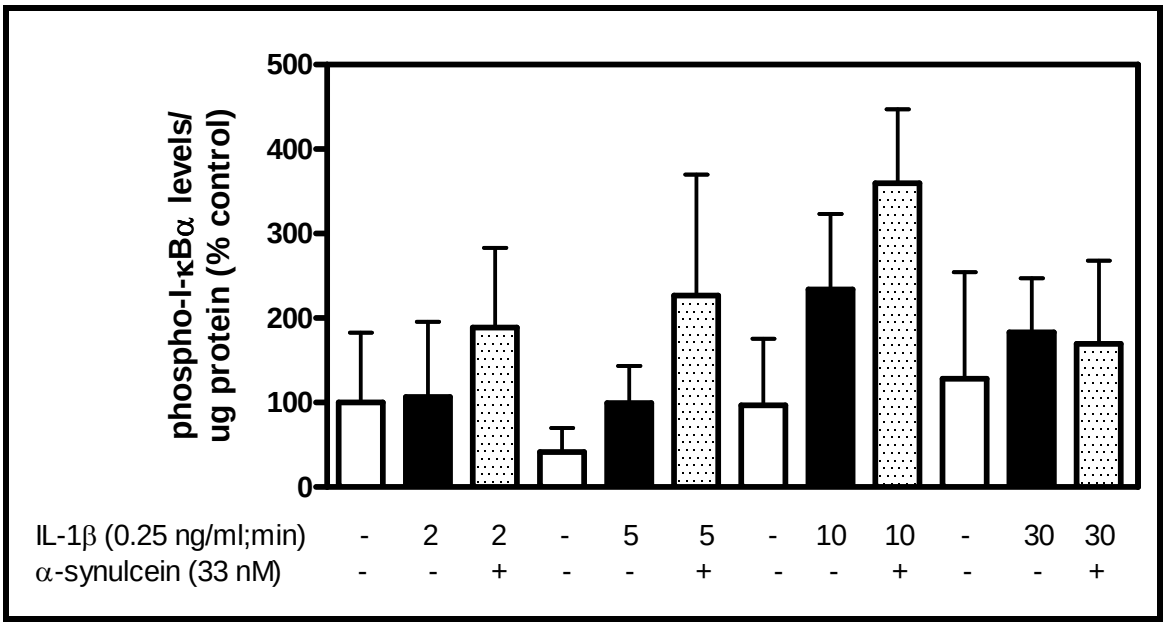



**Figure 21. Effects of α-synuclein on phosphorylated I-κBα levels in human A172 astroglial cells.** Cells were exposed to α-synuclein in serum-free medium for 24 h. Also, during the last 2, 5, 10, and 30 min, cells were co-exposed to IL-1β (0.25 ng/ml). No significant enhancement in the levels of phosphorylated I-κBα in A172 cells were observed in the presence of IL-1β relative to unstimulated cells. Astroglial cells co-exposed with both IL-1β and α-synuclein did not result in a significant modulation of phosphorylated I-κBα levels relative to IL-1β-induced levels. Levels of phosphorylated I-κBα were quantitated by ELISA. Data were normalized as percent control within each independent experiment and represent the mean + S.E.M. of duplicate measures from 1-3 independent experiments. No significant differences ($P$>0.05) among treatment were determined by one-way ANOVA.

### 4.7.2 Alpha-synuclein does not alter NF-κB activation

Further examination of the effects of α-synuclein on NF-κB signaling in A172 cells was investigated via the levels of active nuclear NF-κB p65 and p50 proteins (Figure 22). Nuclear extracts of NF-κB p65 and p50 were at basal levels in unstimulated astroglial cells (Figure 22A). The addition of IL-1β to A172 cells enhanced p50 nuclear levels and greatly potentiated p65 nuclear levels. The induction of active NF-κB proteins by IL-1β was observed by 30 minutes and resulted in a peak increase in p65 protein by 60 minutes. Results on active p65/p50 from A172 cells co-exposed with α-synuclein and IL-1β overlap results from IL-1β-only treatments and therefore, present no significant fluctuations by α-synuclein on NF-κB proteins compared to IL-1β alone (Figure 22B).

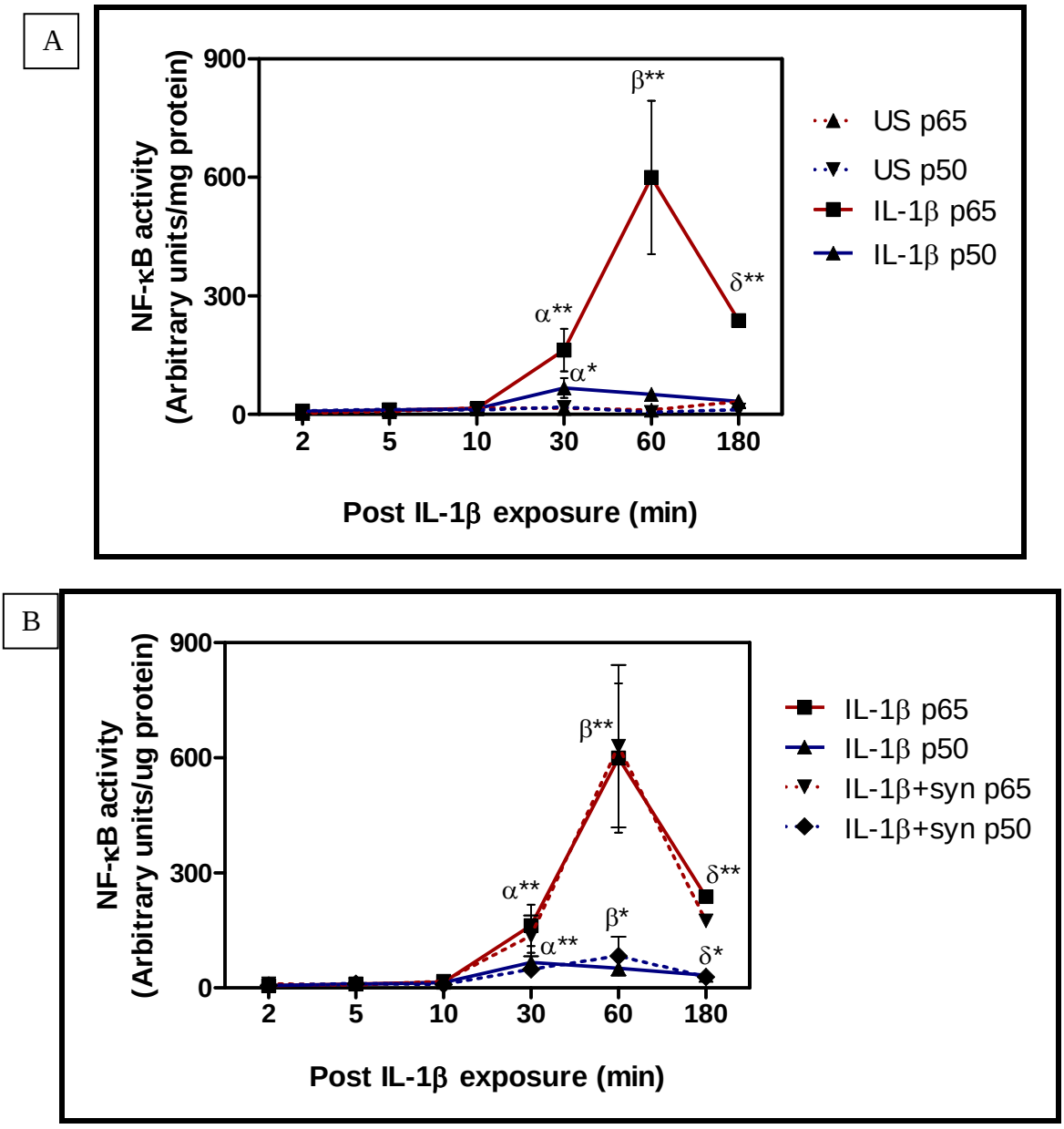



**Figure 22. Alpha-synuclein did not alter IL-1β-induced increases in nuclear levels of active NF-κB signaling proteins in human A172 astroglial cells.** Cells were unstimulated or exposed to α-synuclein for 24 h in serum-free medium and co-exposed to IL-1β (0.25 ng/ml) during the last 2-180 minutes of treatment. Nuclear extracts of NF-κB p65 and p50 were at basal levels in unstimulated astroglial cells, and the addition of IL-1β enhanced p50 nuclear levels and greatly potentiated p65 nuclear levels (A). Levels of active p65/p50 from A172 cells co-exposed with α-synuclein and IL-1β overlap results from IL-1β-only treatments and reveal no significant fluctuations by α-synuclein on NF-κB proteins compared to IL-1β alone (B). Arbitrary binding activity of NF-κB p65 and p50 signaling proteins were determined chemiluminescently using nuclear extracts. Data presented represent the mean + S.E.M. of duplicate measures from 2-4 independent experiments. Significant differences among treatments were determined by one-way ANOVA with Newman-Keul's post-doc comparisons. Letters and asterisks represent statistical differences in (A) IL-1β and (B) IL-1β + syn treatments. *P<0.05, **P<0.01, α = 10 vs. 30 minutes, β = 30 vs. 60 minutes, δ = 60 vs. 180 minutes.

## 4.8 Supplementary Data: Alpha-synuclein does not modulate IL-1β-induced CXCL10 protein expression in Normal Human Astrocytes

Concurrent to investigating the effects of α-synuclein in human A172 cells, experiments examining the effects of α-synuclein in NHA were also investigated. Specifically, α-synuclein's effect on CXCL10 protein expression was examined in NHA (Figures 23A and B), with observations resulting in minimum protein induction in unstimulated cells and an up-regulation of CXCL10 expression after a 24-hour treatment of IL-1β (0.1-5 ng/ml). The addition of α-synuclein (3.7, 11, 33 nM) to IL-1β-stimulated NHA cells did not result in significant CXCL10 protein modulations compared to IL-1β-induced CXCL10 protein levels (Figure 23A and B).

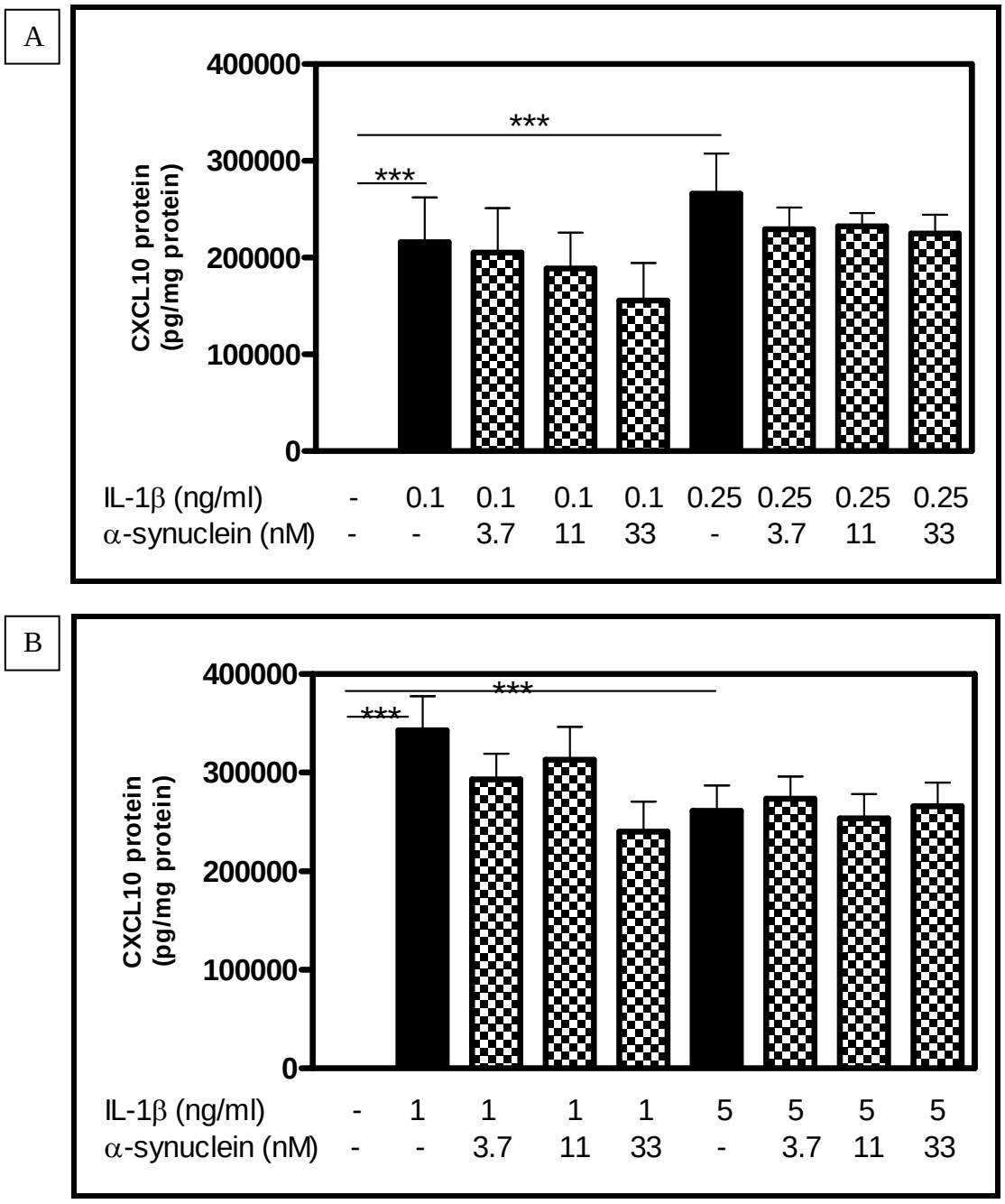



**Figure 23. Effect of α-syncuelin on IL-1β-induced CXCL10 expression in NHA cells.** Cells were exposed to IL-1β at (A) 0.1 and 0.25 ng/ml or (B) 1 and 5 ng/ml concentrations in the presence or absence of α-synuclein (3.7,11,33 nM) in serum-free medium for 24 h. Minimum CXCL10 protein induction was observed in unstimulated cells, with an up-regulation of chemokine expression after a 24-hour treatment of IL-1β (0.1-5 ng/ml) (A and B). NHA cells co-exposed to α-synuclein (3.7, 11, 33 nM) and IL-1β did not result in significant CXCL10 protein modulations compared to IL-1β-induced CXCL10 protein levels (A and B). Levels of secreted CXCL10 protein in the media were quantitated by ELISA. Data presented represent the mean + S.E.M. of triplicate measures from 3-4 independent experiments. Significant differences were determined by one-way ANOVA with Dunnett's post-hoc comparisons. ***$P < 0.05$ vs control.

## Part III

## Neuromelanin and oxidized dopamine modulation of inflammatory signaling in TNFα-stimulated human A172 astroglial cells

### 4.9 Neuromelanin and oxDA inhibit CXCL10 expression in A172 astroglial cells

The effect of other PD-associated molecules, NM and oxDA, were also examined in human A172 astroglial cells in this study. When A172 cells were unstimulated, cells produced constitutive levels of CXCL10 protein (Figure 24). Also, cells exposed to NM- or oxDA-only treatments did not produce altered chemokine levels relative to unstimulated cells (Figure 24A and B). However, consistent with Figures 8 and 10, TNFα (5 ng/ml) significantly enhanced the expression of CXCL10 protein in A172 cells. Treatment groups of ≤ 3.7 μM oxDA with TNFα did not affect TNFα-induced CXCL10 production (Figure 24A); however, a significant reduction (75%) in CXCL10 expression was observed in response to 300 μM oxDA relative to TNFα alone. Cytotoxicity in A172 astroglial cells was observed at 300 μM in the presence of TNFα but resulted in only a 10% decrease in viability (Figure 25A). Nonetheless, the reduction in CXCL10 protein expression by 300 μM oxDA may, in part, be due to less viable cells. Significant reduction in protein expression was also observed in A172 cells exposed to 75 and 150 μM oxDA in the presence of TNFα, with no alterations on cell viability (Figure 25A).

Slight differences in CXCL10 production were observed at ≤ 5 μg/ml NM (Figure 24B); however, only15 μg/ml NM caused a significant reduction (30%) in CXCL10 levels. No significant alterations in astroglial cell viability were seen from NM treatments (Figure 25B).

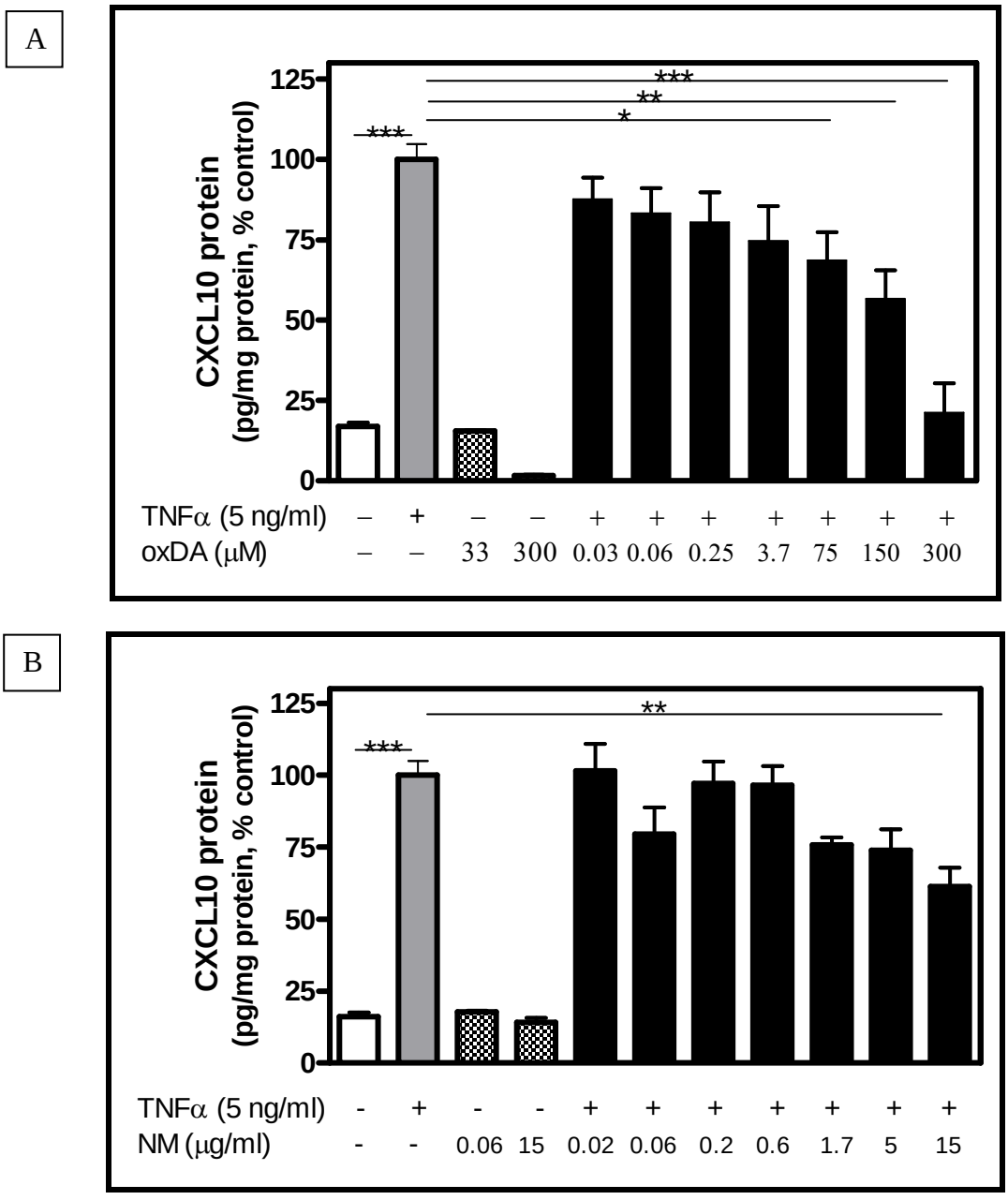



**Figure 24. Effects of oxidized dopamine (oxDA) and neuromelanin (NM) on TNFα-induced CXCL10 expression in A172 cells.** Cells were exposed to (A) oxDA or (B) NM in serum-free medium for 72 h. Also, during the last 24 h, cells were co-exposed to TNFα (5 ng/ml). Unstimulated A172 cells produced constitutive levels of CXCL10 protein (A and B) and neither NM nor oxDA-only treatments altered the production (A and B). TNFα (5 ng/ml) did enhance the expression of CXCL10 protein (A and B), with a significant reduction in CXCL10 expression observed at 300 μM oxDA (A) and 15 μg/ml NM (B) relative to TNFα alone. Levels of secreted CXCL10 protein in the media were quantitated by ELISA. Data were normalized as percent control within each independent experiment and represent the mean + S.E.M. of triplicate measures from 3-4 independent experiments. Significant differences were determined by one-way ANOVA with Dunnett's post-hoc comparisons. *$P < 0.05$ vs TNFα; **$P < 0.01$ vs TNFα, ***$P < 0.001$ vs TNFα.

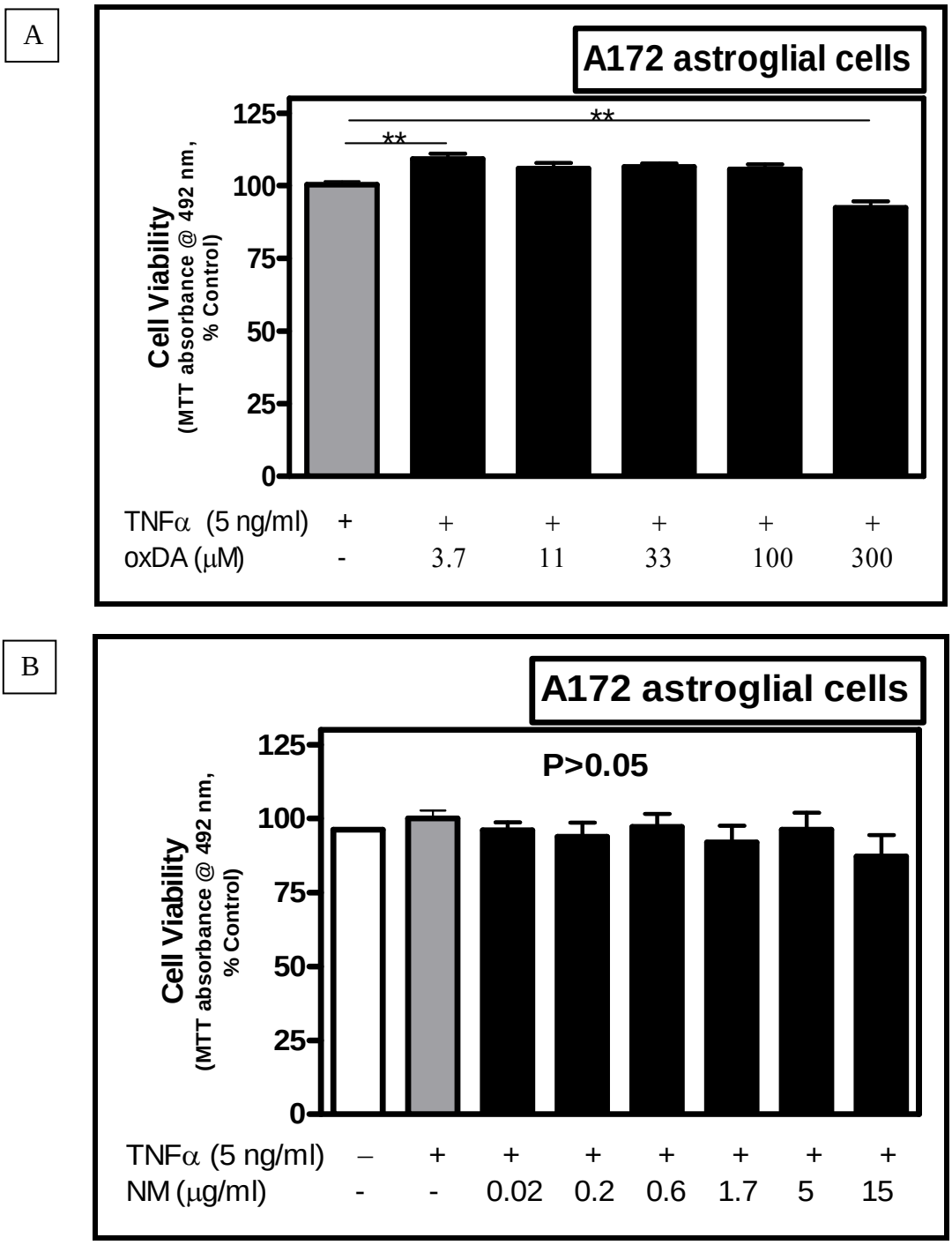



**Figure 25. Oxidized dopamine- and neuromelanin-induced cytotoxicity in TNFα-stimulated human astroglial cells.** Cells were exposed to (A) oxDA or (B) neuromelanin in serum-free medium for 72 h. Also, during the last 24 h, cells were co-exposed to TNFα (5 ng/ml). Cytotoxicity in A172 astroglial cells was observed at 300 μM oxDA in the presence of TNFα (A). NM treatments did not significantly alter astroglial cell viability, as seen in (B). Cell viability was assessed using the MTT assay. Data were normalized as percent control within each independent experiment and represent the mean + S.E.M. of 2-4 measures from 2-5 independent experiments for each cell type. Significant differences were determined by one-way ANOVA with Newman-Keul's post-hoc comparisons. $^{**}P < 0.01$ vs TNFα.

### 4.10 Neuromelanin and oxDA down-regulate NF-κB activation in TNFα-stimulated A172 astroglial cells

The activation of NF-κB was assessed by the nuclear translocation of the NF-κB proteins p65 and p50. Examination of the effects of TNFα on NF-κB activation was conducted to determine the optimal time for NF-κB activation by TNFα for subsequent experiments. Nuclear levels of NF-κB were used as an indicator of NF-κB activation. As seen in Figure 26, TNFα potentiated the levels of NF-κB in a time-dependent manner in A172 cells. Specifically, NF-κB activity initially peaked at 10 minutes, with elevation seen at 3 minutes and reduction at 810 minutes.

Next, the examination of the effects of oxDA and NM on NF-κB activation in A172 cells was observed (Figures 27 and 28). Induction of NF-κB activation by the cytokine TNFα at a median peak time of 30 minutes was examined in the presence of oxDA and NM (Figures 27 and 28). Activation of NF-κB was not affected by oxDA or NM alone compared to the unstimulated cells as indicated by no significant change in nuclear levels of NF-κB (Figures 27 and 28). Conversely, oxDA decreased TNFα-induced NF-κB activation in astroglial cells between 60-75% relative to TNFα alone (Figure 27). However, the reduction in activation may be due to a decrease in viable cells by 300 μM oxDA (Figure 25A). Neuromelanin also decreased TNFα-induced NF-κB activation in A172 cells, as presented by a reduction in nuclear p65 levels (Figure 28A), yet similar reduction was not observed for nuclear p50 levels (Figure 28B).

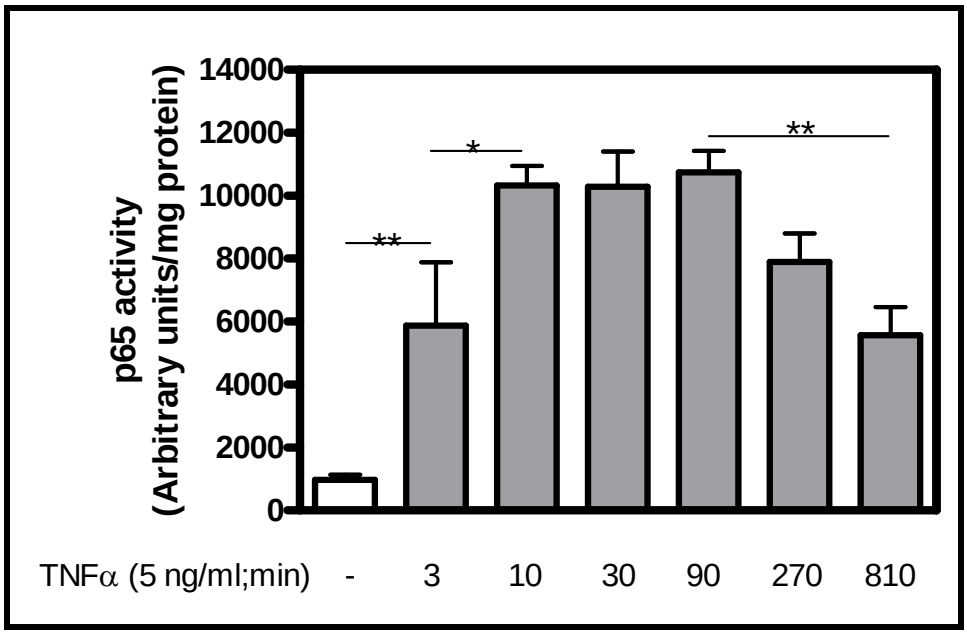
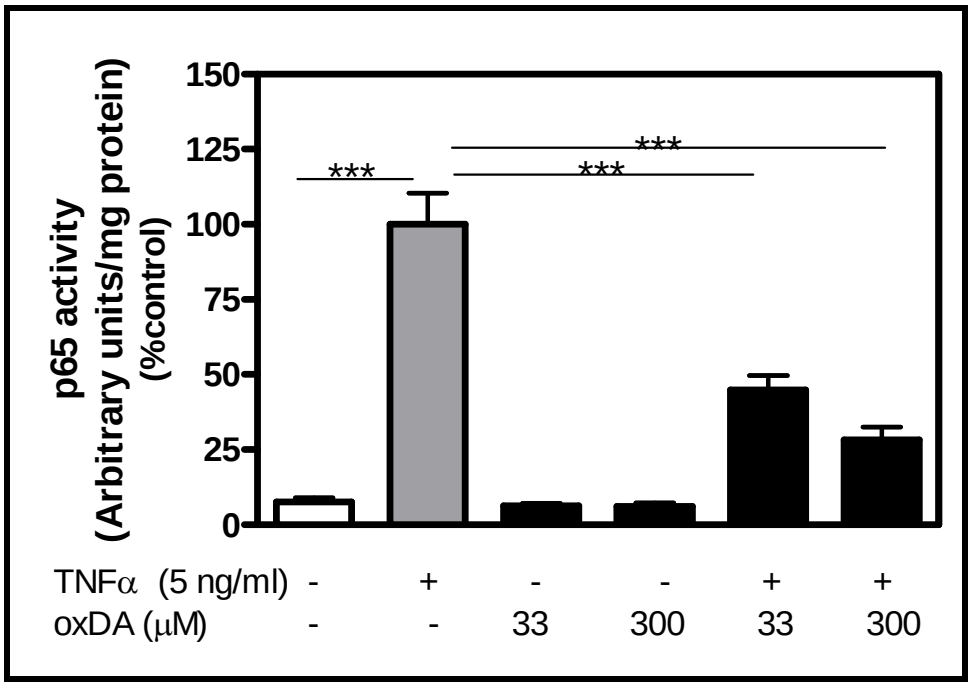



**Figure 26. Time-dependent response of TNFα to active NF-κB p65 signaling protein in human A172 astroglial cells.** Cells were exposed to TNFα (5 ng/ml) in serum-free medium for the time course ranging from 3 to 810 minutes. TNFα potentiated the levels of NF-κB in a time-dependent manner in A172 cells, with elevation seen at 3 minutes, peaks from 10-90 minutes, and reduction at 810 minutes. Arbitrary binding activity of NF-κB p65 and p50 signaling proteins were determined chemiluminescently using nuclear extracts. Data presented represent the mean + S.E.M. of duplicate measures from 2-4 independent experiments. Significant differences were determined by one-way ANOVA with Dunnett's post-hoc comparisons. $*P < 0.05$ vs TNFα, $**P < 0.01$ vs TNFα.

A




B

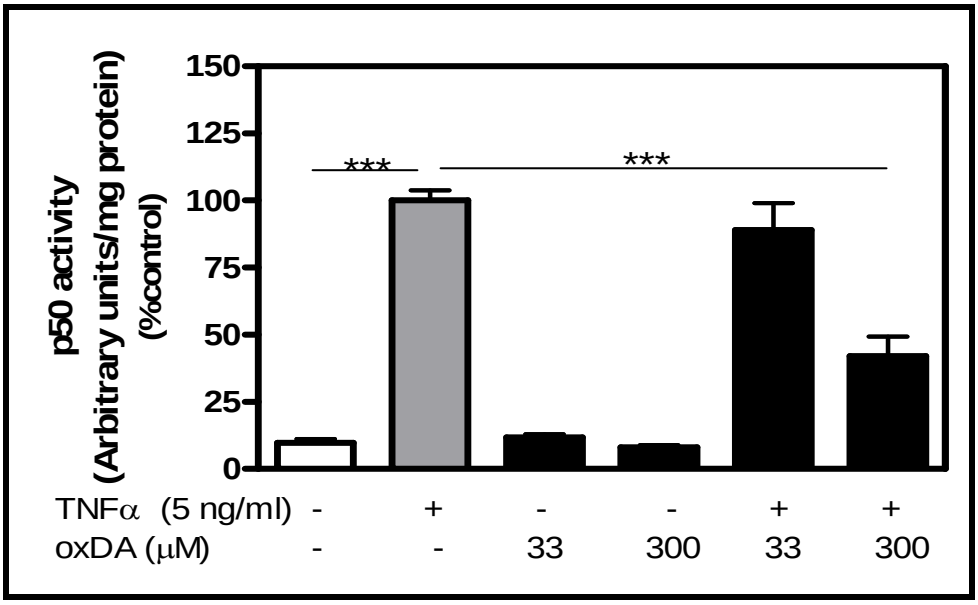



**Figure 27. Oxidized dopamine decreased TNFα-induced NF-κB activation in human A172 astroglial cells.** Cells were exposed to oxDA in serum-free medium for 72 h. Also, during the last 30min, cells were co-exposed to TNFα (5 ng/ml). Activation of NF-κB was not affected by oxDA alone compared to unstimulated cells (A and B), however, oxDA decreased TNFα-induced NF-κB activation in astroglial cells between 60-75% relative to TNFα alone (A and B). NF-κB activation was assessed by using transcription factor assay which measures levels of active NF-κB. Arbitrary binding activity of NF-κB p65 and p50 signaling proteins were determined chemiluminescently using nuclear extracts. Data were normalized as percent control within each independent experiment and represent the mean + S.E.M. of duplicate measures from two independent experiments. Significant differences were determined by one-way ANOVA with Dunnett's post-hoc comparisons. ***$P < 0.001$ vs TNFα.

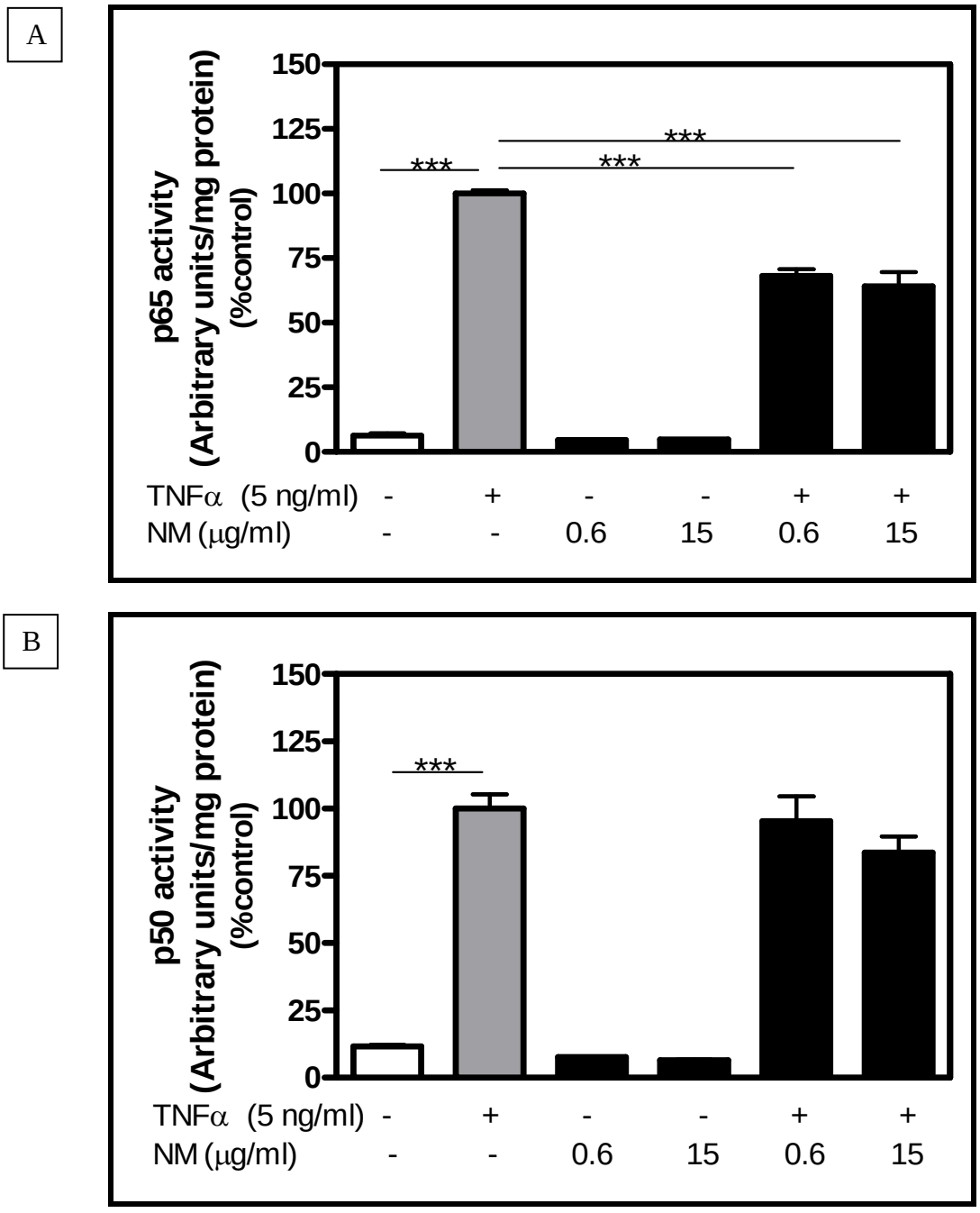



**Figure 28. Neuromelanin reduced TNFα-induced NF-κB activation in human A172 astroglial cells.** Cells were exposed to NM in serum-free medium for 72 h. Also, during the last 30 minutes, cells were co-exposed to TNFα (5 ng/ml). NF-κB activation was not affected by NM alone compared to the unstimulated cells, as seen in A and B. However, NM in the presence of TNFα decreased TNFα-induced nuclear p65 levels (A), yet similar reduction was not observed for nuclear p50 levels (B). NF-κB activation was assessed by using transcription factor assay which measures levels of active NF-κB. Arbitrary binding activity of NF-κB p65 and p50 signaling proteins were determined chemiluminescently using nuclear extracts. Data were normalized as percent control within each independent experiment and represent the mean + S.E.M. of duplicate measures from 2 independent experiments. Significant differences were determined by one-way ANOVA with Dunnett's post-hoc comparisons. ****P* < 0.001 vs TNFα.

## 4.11 Supplementary data:

### 4.11.1 Oxidized dopamine- and neuromelanin-induced cytotoxicity in human neuronal and/or glial cells.

In this inflammatory model where cells were co-exposed to oxDA and TNFα, oxDA was more toxic to SK-N-SH neuronal cells than to glia (Figure 29). As shown in Figure 29A, ≥ 300 μM oxDA was cytotoxic (approximately 45-75% decrease in viability) in neuronal cells compared to TNFα alone. Furthermore, in CHME-5 microglia cells, cytotoxicity was observed with a 20% reduction in viability in cultures exposed to ≥ 300 μM (Figure 29B).

When cells were exposed to NM with co-exposure to TNFα, NM was more toxic to SK-N-SH neuronal cells than to glia (Figure 30). The number of viable SK-N-SH cells compared to TNFα alone was significantly reduced by ≥ 5 μg/ml NM (Figure 30A). At the highest concentration of NM tested (15 μg/ml), there was a 40% decrease in viable SK-N-SH cell number. In comparison, exposure to NM (0.02-15 μg/ml) did not significantly alter viability in CHME-5 cultures (Figure 30B).

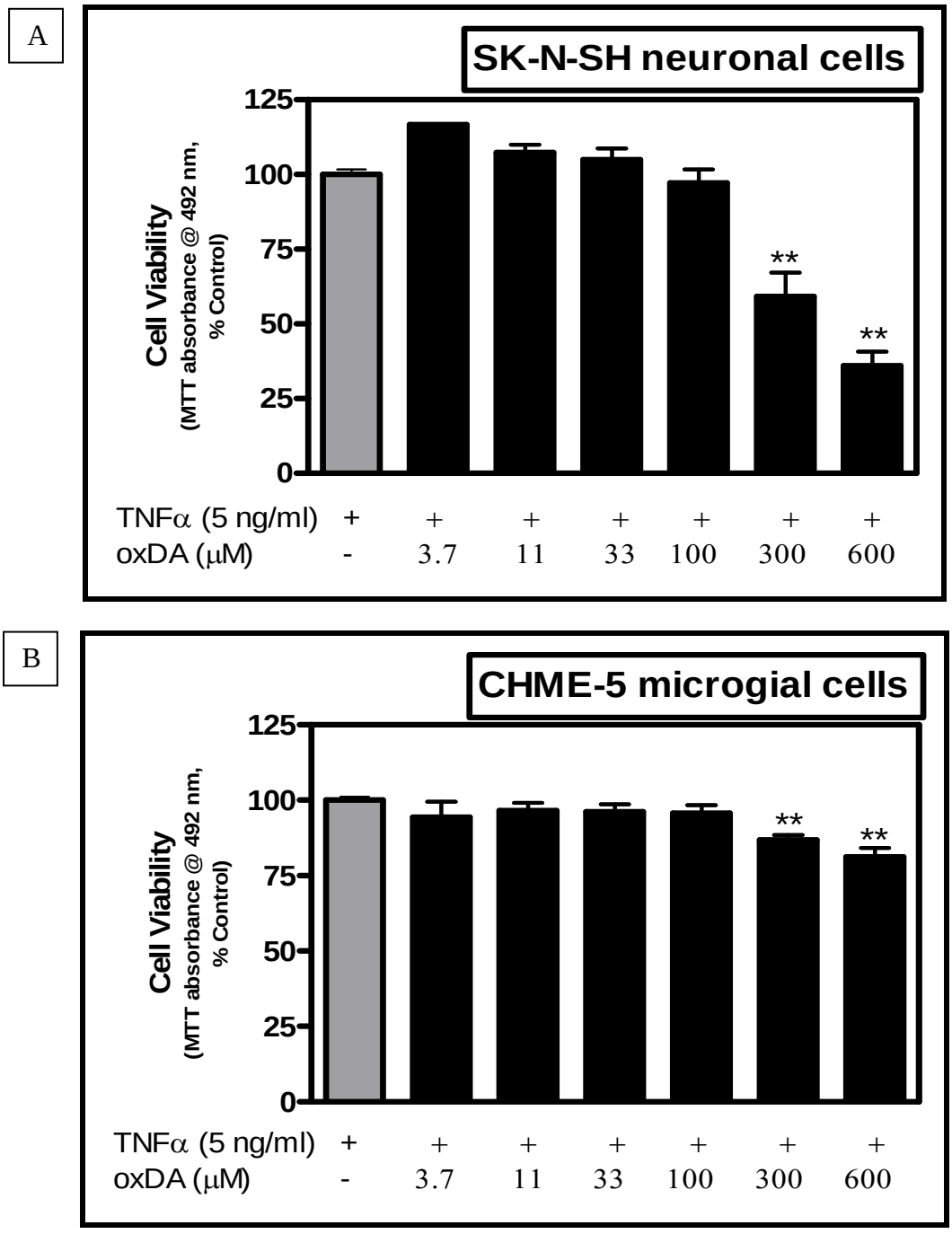



**Figure 29. Oxidized dopamine-induced cytotoxicity in human neuronal and glial cells.** Cells were exposed to oxDA in serum-free medium for 72 h. Also, during the last 24 h, cells were co-exposed to TNFα (5 ng/ml). oxDA concentrations of ≥ 300 μM were found to be cytotoxic to neuronal cells (45-75% decrease, A) and CHME-5 microglial cells (20% reduction, B) compared to TNFα alone. Cell viability was assessed using the MTT assay. Data were normalized as percent control within each independent experiment and represent the mean + S.E.M. of 2-4 replicate measures from 4-5 independent experiments for each cell type. Significant differences were determined by one-way ANOVA with Newman-Keul's post-hoc comparisons. $^{**}P < 0.01$ vs TNFα.

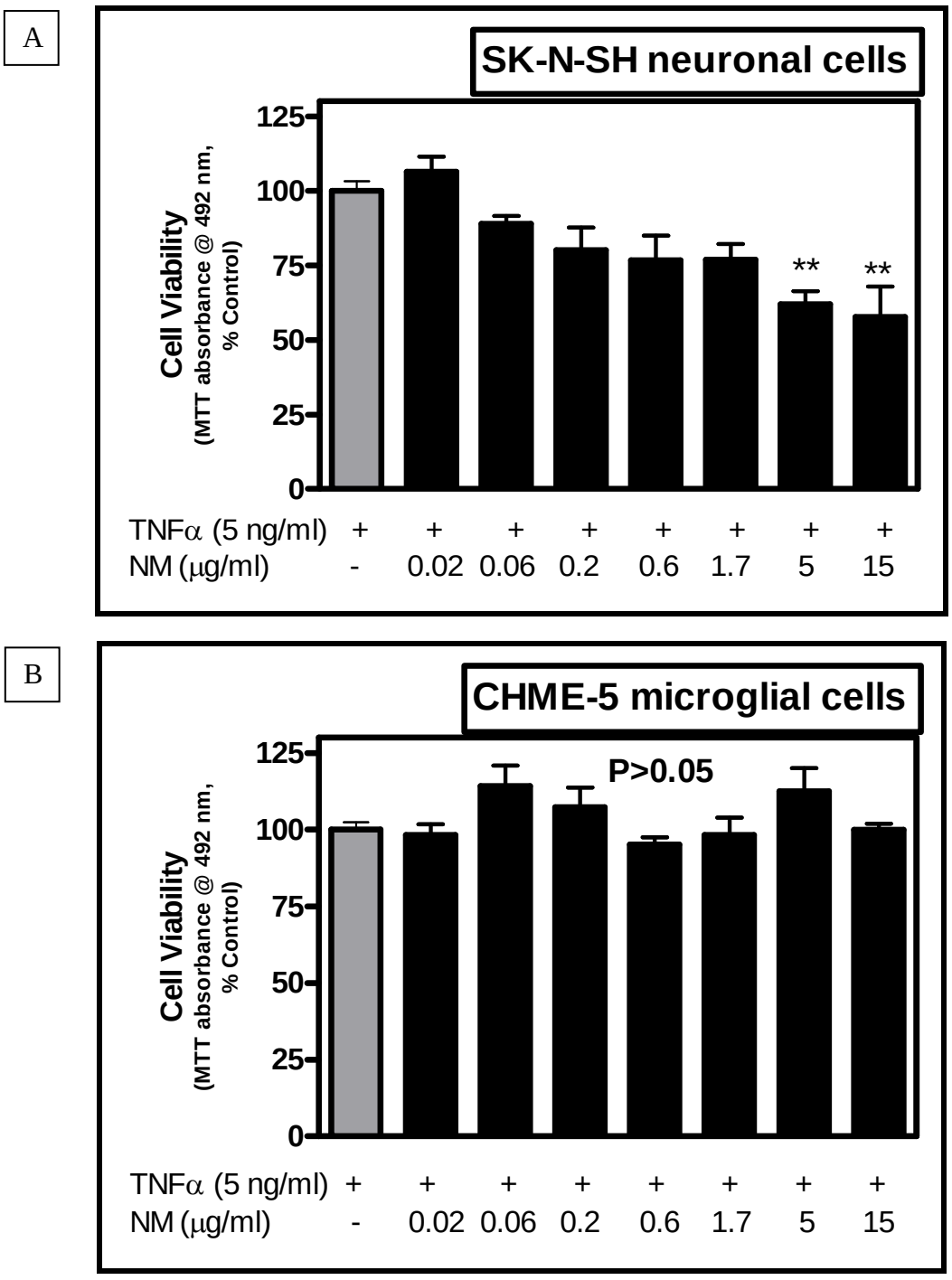



**Figure 30. Neuromelanin-induced cytotoxicity in TNFα-stimulated human neuronal and glial cells.** Cells were exposed to neuromelanin in serum-free medium for 72 h. Also, during the last 24 h, cells were co-exposed to TNFα (5 ng/ml). For neuronal cells, the number of viable SK-N-SH cells compared to TNFα alone was significantly reduced (40% decrease) by ≥ 5 μg/ml NM (A). Exposure to NM (0.02-15 μg/ml) did not significantly alter viability in CHME-5 cultures (B). Cell viability was assessed using the MTT assay. Data were normalized as percent control within each independent experiment and represent the mean + S.E.M. of triplicate measures from 2-3 independent experiments for each cell type. Significant differences were determined by one-way ANOVA with Newman-Keul's post-hoc comparisons. ***P* < 0.01 vs TNFα.

### 4.11.2 Oxidized dopamine and neuromelanin enhance TNFα-induced iNOS induction in human astroglial cells

To determine the effects of oxDA and NM on iNOS induction, nitrite accumulation was used as an indicator of iNOS activity. Minimal nitrite production was detected from A172 cells by TNFα-alone treatments (Figure 31A) and by oxDA- and NM-alone treatments (data not shown). The effect of oxDA on cytokine mixture-induced nitrite production was observed and resulted in no significant modulation of iNOS induction relative to cytokine mixture alone (Figure 31B). Conversely, oxDA at ≥ 100 μM significantly potentiated TNFα-induced iNOS induction (Figure 31A). Similarly, induction of iNOS was potentiated by 0.2-5 μg/ml NM, however lower (0.06 μg/ml) and higher (15 μg/ml) NM concentrations had minimal effect on iNOS induction (Figure 32).

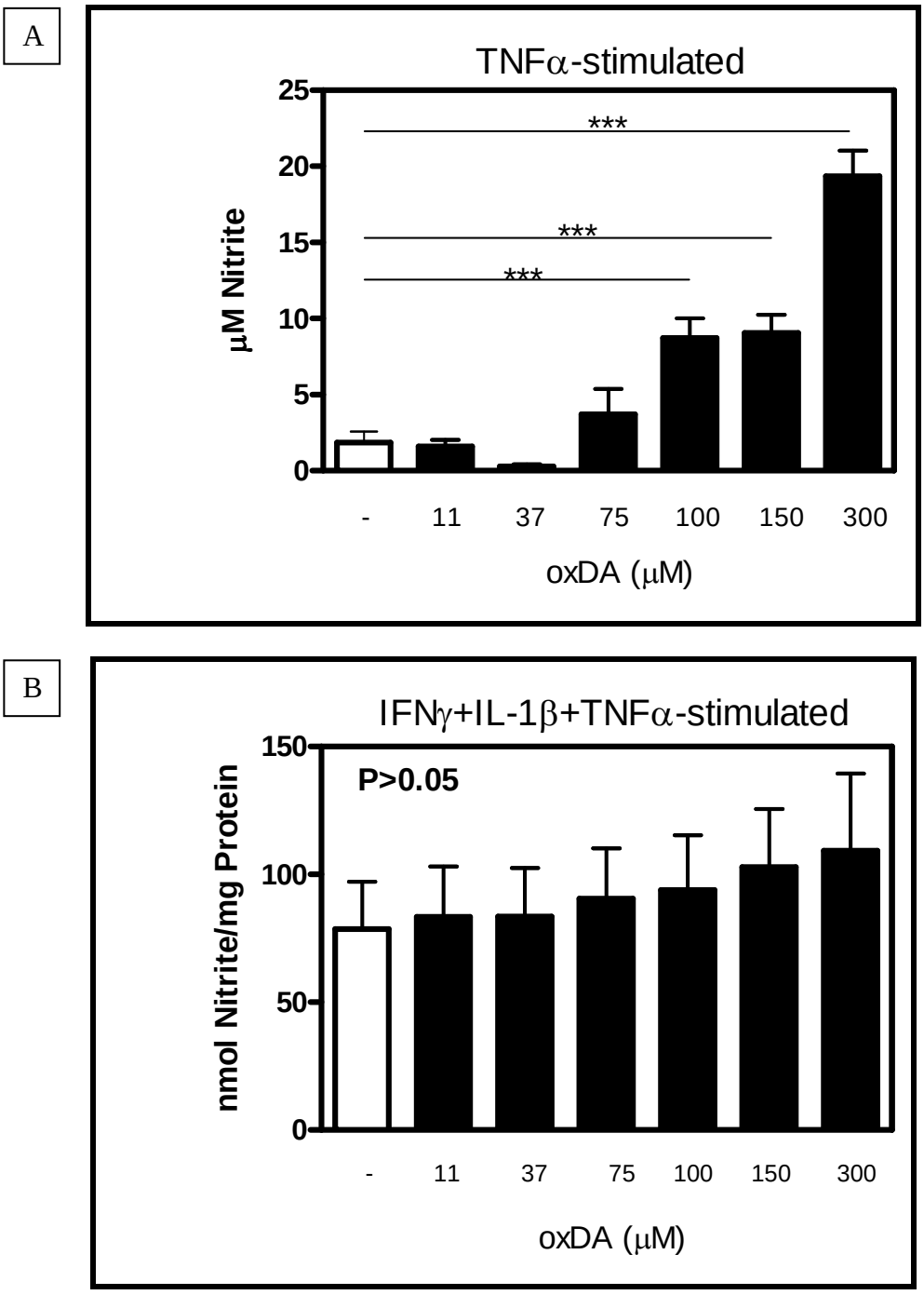



**Figure 31. Oxidized dopamine enhances TNFα-induced nitrite production in human A172 astroglial cells.** Cells were exposed to oxDA for 72 h. Also, during the last 24 h, cells were co-exposed to (A) TNFα (5 ng/ml) or (B) a cytokine mixture containing TNFα (30 ng/ml), IFNγ (100 ng/ml), and IL-1β (5 ng/ml). Nitrite production from A172 cells was minimal by TNFα-alone treatments (A), however, oxDA at ≥ 100 μM significantly potentiated TNFα-induced iNOS induction (A). The effect of oxDA on cytokine mixture-induced nitrite production resulted in no significant modulation of iNOS induction relative to cytokine mixture alone (B). Nitrite accumulation in the culture media was determined spectrophotometrically using the Griess reagent. Data represent the mean + S.E.M. of triplicate measures from 2-4 independent experiments. Significant differences were determined by one-way ANOVA with Dunnett's post-hoc comparisons. ***$P < 0.001$ vs TNFα.

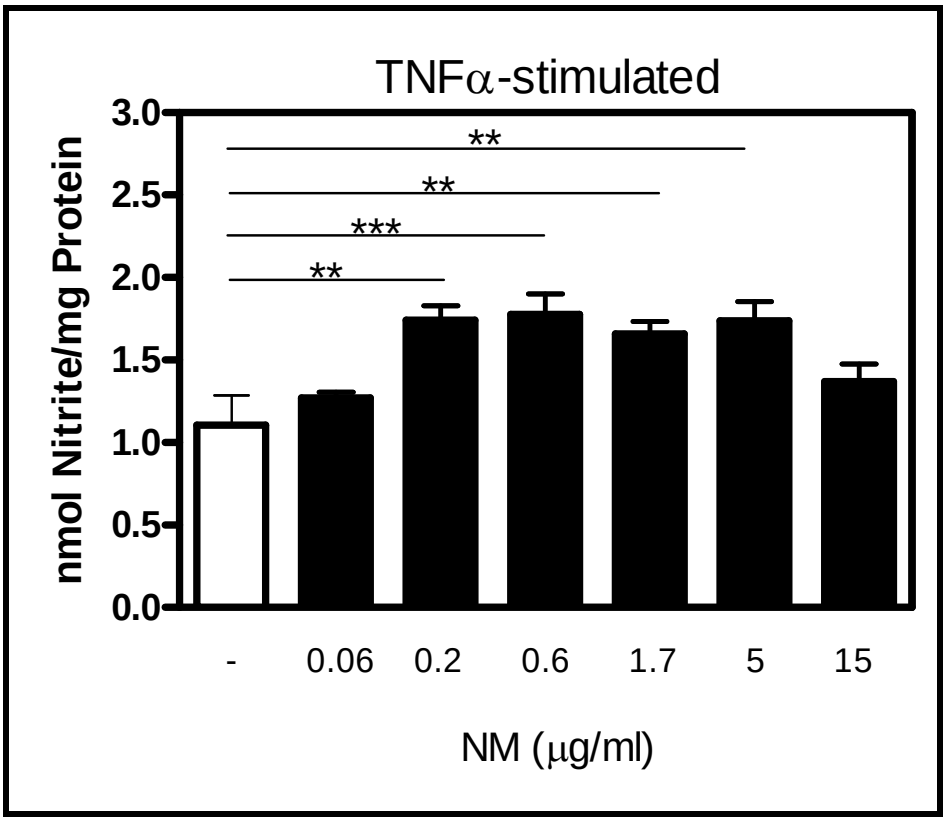



**Figure 32. Neuromelanin enhances TNFα-induced nitrite production in human A172 astroglial cells.** Cells were exposed to NM for 72 h. Also, during the last 24 h, cells were co-exposed to TNFα (5 ng/ml). Although the lower (0.06 μg/ml) and higher (15 μg/ml) NM concentrations had minimal effect on iNOS induction , 0.2-5 μg/ml NM potentiated iNOS expression relative to TNFα alone. Nitrite accumulation in the culture media was determined spectrophotometrically using the Griess reagent. Data represent the mean + S.E.M. of quadruplicate measures from 3 independent experiments. Significant differences were determined by one-way ANOVA with Dunnett's post-hoc comparisons. $^{**}P < 0.01$ vs TNFα, $^{***}P < 0.001$ vs TNFα.

# CHAPTER V

# DISCUSSION

## Part I

## Alpha-synuclein modulation of inflammatory signaling in cytokine-stimulated human A172 astroglial cells

### 5.1 Alpha-synuclein enhanced cytokine-stimulated CXCL10 expression in human A172 astroglial cells

Parkinson's disease studies reveal an up-regulation of α-synuclein with extensive oligomeric forms elevated in the cerebrospinal fluid and blood plasma of PD patients compared to healthy controls (El-Agnaf et al., 2003; El-Agnaf et al., 2006). Neuroinflammation is also observed in PD, with an increase in proinflammatory cytokines and chemokines (Forno et al., 1992; Hunot and Hirsch, 2003; Miklossy et al., 2006). Astrocytes are the major cellular source of chemokine production in the CNS and demonstrate increased reactive astrocytosis in PD (Teismann and Schulz, 2004). Understanding α-synuclein's effects on astrocytes in the presence of on-going inflammation is therefore potentially important. The two-hit (neuroinflammation and α-synuclein) *in vitro* model created for this study mimics PD etiology and produces novel attributes to PD research.

First, the role of α-synuclein aging was examined to obtain information on the length of time for aggregation for astroglial studies. Initial studies determined that 7-day aggregation was not sufficient to significantly modulate TNFα-induced chemokine protein expression. The current data suggest that longer than seven days is needed to generate a molecule that is proinflammatory in this A172 model. This is consistant with a study by Zhang and colleagues (2005), who characterized aged α-synuclein by a size exclusion column. Zhang et al. (2005) determined the nature of human α-synuclein and found 7 days of aging formed α-synuclein aggregates between 44 and 158 kDa, which is consistent with a soluble oligomer conformation. Fourteen days of aging formed α-synuclein aggregates sized > 670 kDa, which represent fibrillary α-synuclein conformation (Zhang et al., 2005). Although oligomer α-synuclein is more toxic to neurons than fibrillary polymers, Lewy bodies (which are the pathological hallmark of PD) are primarily composed of fibrillary α-synuclein (Spillantini et al., 1998). Therefore, the current results suggest the change in inflammatory property in human A172 astroglial cells may be potentiated by the presence of α-synuclein fibrillary polymers.

The present data is the first report examining the role of extracellular, aggregated α-synuclein on human astroglial cells. Only one other study has explored the effects of α-synuclein on CXCL10 protein expression, yet this was studied in mouse mixed glia and isolated microglia cultures (Roodveldt et al., 2010). The same group found CXCL10 protein levels produced by unstimulated cells and wild-type α-synuclein-alone treated cells to be at a low level in both microglia-astroglia mixed culture and the isolated microglia culture (Roodveldt et al., 2010). The low expression of CXCL10 by glial cells in α-synuclein treatments is similarly demonstrated in the present data. On the other

hand, several studies reveal α-synuclein's ability to enhance production of other inflammatory factors (Gao et al., 2011; Gu et al., 2010; Reynolds et al., 2008a). For example, α-synuclein significantly increased the levels of MCP-1, MIP-1, and RANTES in microglial cells and mixed astroglia-microglia co-culture (Reynolds et al., 2008a; Roodveldt et al., 2010). Klegeris et al. (2006) reported that α-synuclein stimulated human astrocytes as well as human astrocytoma cells to up-regulate both IL-6 and ICAM-1. The results, however, were obtained from studies that demonstrated several differences compared to the current study, including cell- and species-specific differences and α-synuclein conformational differences. Although previous studies reveal a regulation of inflammatory factors by α-synuclein on glial cells, outcomes from the present study agree with Roodveldt et al. (2010), which indicate that α-synuclein alone is not able to increase the production of CXCL10.

Results of enhanced glial CXCL10 protein expression by the cytokines in the current study are in agreement with other studies (Davis et al., 2007; John et al., 2001; Qi et al., 2009). For instance, IL-1β induced the expression of CXCL10 protein in human fetal astrocytes (John et al., 2001). In a study conducted by the current laboratory, published results revealed an enhanced CXCL10 expression by TNFα and IL-1β in human A172 astrocytes relative to unstimulated cells (Davis et al., 2007). Overall, our results correlate with others and state that TNFα and IL-1β are capable of stimulating the production of CXCL10 in astroglial cells.

Examining α-synuclein in the presence of either TNFα or IL-1β was found to up-regulate cytokine-induced CXCL10 expression in A172 cells. This is the first study to examine the effect of α-synuclein in the presence of either cytokine on CXCL10 protein

expression in human astroglial cells. Interestingly, few other reports have examined the combination of α-synuclein and inflammatory factor(s) on the alterations of cytokines/chemokines (Gao et al., 2011; Klegeris et al., 2006). For example, Klegeris et al. (2006) studied the *in vitro* expression of IL-6 and ICAM-1 induced by α-synuclein in astroglia. Results revealed that the addition of α-synuclein and IFNγ to human astrocytes and astrocytoma cells resulted in an increase in ICAM-1 and IL-6 levels. Additionally, Gao et al. (2011) examined the effects of α-synuclein with the TLR4 ligand lipopolysaccharide (LPS) on neuroinflammation in brains of mice. Specifically, Gao and colleagues (2011) detected an up-regulation of multiple inflammatory markers, including Iba-1, Mac1, iNOS, and cyclooxygenase-2 (COX-2) in both the SN and striatum of transgenic mice overexpressing human A53T mutant α-synuclein. The up-regulation of inflammatory markers was relative to LPS-injected wildtype mice and was consistent with chronic progressive degeneration of nigrostriatal dopamine pathway. Examining the effects of α-synuclein on glial CXCL10 expression did not increase the expression of the chemokine at lower TNFα concentrations in the current study. The data indicate that TNFα at 5 ng/ml may provide a threshold level needed for α-synuclein to have an effect on CXCL10 protein expression in A172 cells. Data with IL-1β-induced CXCL10 expression also demonstrated a significant up-regulation by α-synuclein at an IL-1β concentration of 0.25 ng/ml. The result may indicate that maximum CXCL10 production may be achieved at IL-1β greater than 0.25 ng/ml; therefore, not allowing further enhancement of chemokine production by the addition of α-synuclein. Nonetheless, α-synuclein co-exposed with TNFα or IL-1β potentiated the enhancement of CXCL10

expression in human astroglial cells, demonstrating a potential involvement of α-synuclein in PD neuroinflammation.

The consequences of altered CXCL10 expression in dopamine associated neuropathology have not been fully elucidated. Depending on the temporal, regional, and magnitude of expression in the brain, CXCL10 may be neuroprotective or neurotoxic. CXCL10 can display neuroprotective properties via antimicrobial activities (Cole et al., 2001). Neurotoxic behavior of CXCL10 is well documented in neurological diseases (Sui et al., 2004; Sui et al., 2006; Tanuma et al., 2006; van Marle et al., 2004). For instance, a study demonstrated neuronal death caused by the supernatant of HIV-1 Nef exposed astrocytes was blocked by an antibody to the CXCL10 receptor, CXCR3 (van Marle et al., 2004). Another study revealed that cultured human fetal brain neurons treated with CXCL10 activate caspase-3 and ultimately leads to apoptosis (Sui et al., 2004). Although chemokines are elevated in the PD brain (Roodveldt et al., 2010), their consequences on dopaminergic neurons are yet to be determined in PD. Therefore, a strong support of the neurotoxic effects of CXCL10 exists in other neurodegenerative disease, and thus, may have similar outcomes in PD.

## 5.2 Alpha-synuclein did not modulate cytokine-stimulated CXCL10 mRNA in human A172 cells

Examining transcriptional regulation by α-synuclein may provide insight into the mechanisms of α-synuclein induced CXCL10 protein expression in A172 cells. The enhancement of the levels of CXCL10 mRNA by IL-1β in the current study was similarly seen in a study by John et al. (2001). In this study, a group of researchers detected IL-1β-induced expression of CXCL10, as well as IL-8 and MCP-1, mRNA in human primary

astrocyte cultures (John et al., 2001). Furthermore, previous study in A172 cells also demonstrated the ability of IL-1β to trigger CXCL10 mRNA expression; however, cells were also co-exposed with LPS (Davis and Syapin, 2004b). The time-dependent effect of IL-1β on CXCL10 mRNA observed in the present study was also observed by Williams et al. (2009), who revealed a similar time-dependent increase of CXCL10 mRNA levels after IL-1β treatment in A172 cells (Williams et al., 2009a). Overall, results revealed in the current study confirm previous studies and indicate the capability of IL-1β to induce CXCL10 mRNA in A172 cells in a time-dependent manner. The induction of CXCL10 mRNA by IL-1β may be due to signaling pathways that activate transcription factors, such as NF-κB, that facilitate mRNA production, as seen in previous studies (John et al., 2001; Yeruva et al., 2008).

When evaluating the cooperative effects of α-synuclein and IL-1β on CXCL10 mRNA expression, the lack of change by α-synuclein on IL-1β-induced CXCL10 mRNA expression was different compared to the single study that also examined α-synuclein effects on astroglial CXCL10 mRNA (Lee et al., 2010b). Conditioned media from α-synuclein-expressing human neurons transferred to rat astrocytes resulted in an induction of CXCL10, as well as other (CCL2, CCL5, CXCL1, CXCL2), mRNA levels. The study also determined that α-synuclein did not have an effect on astroglial mRNA levels of a particular chemokine (CCL6) and down-regulated mRNA levels of other mediators (Lee et al., 2010b). The dissimilarity in results between the current study and Lee et al. may be due to species-specific differences. Furthermore, while human α-synuclein was aged in the present study to obtain aggregated conformation, neuronal cells used by Lee et al. (2010) were transfected with adenoviral vector containing human α-synuclein cDNA.

The difference in α-synuclein preparation may have different cellular and molecular effects in astrocytes. Overall, the current results suggest that α-synuclein does not mediate the induction of IL-1β-induced CXCL10 mRNA expression. Several possibilities could be responsible for this effect by α-synuclein, including structural modifications of α-synuclein. Alpha-synuclein possesses conformational plasticity and the structure of this protein depends on its environment (Uversky 2007), implying that α-synuclein may undergo structural modifications that do not exhibit regulation of the CXCL10 mRNA expression. Furthermore, Jin et al. (2011) recently demonstrated that α-synuclein mechanistically regulated PKCδ gene expression by negatively inhibiting NF-κB activity and reducing p300 (transcriptional co-activator) levels. However, the reduction of PKCδ was examined in dopaminergic neurons transfected with α-synuclein. Exogenous α-synuclein may portray different effects in astroctes relative to neurons, ultimately resulting in a lack of induction of IL-1β-stimulated CXCL10 mRNA, although further research is needed. The enhancement of CXCL10 protein expression observed in A172 cells by α-synuclein may therefore potentially be elicited by post-transcriptional modifications of α-synuclein.

### 5.3 Alpha-synuclein induced cytokine-stimulated CXCL10 mRNA stability

Post-transcriptional control of gene expression through mRNA transcript stability is important in the regulation of inflammatory genes, particularly of those encoding cytokine and chemokines (Sun and Ding, 2006). CXCL10, in particular, has a transcript half-life that is rather short (about 30 minutes) under resting conditions (Shanmugam et al., 2006). In order to examine transcript stability, the addition of *de novo* transcription

inhibitors was studied in culture. The DNA-dependent RNA synthesis inhibitor actinomycin D is a widely used inhibitor in mRNA transcription stability studies (Dhillon et al., 2007; Galbis-Martinez et al., 2010; Okamoto et al., 2008; Shanmugam et al., 2006). Mechanistically, actinomycin D binds DNA at the transcription initiation complex of genes and prevents transcript elongation by RNA polymerase. Therefore, assessment of whether the actinomycin D – DNA binding sequence is present in the CXCL10 DNA sequence was made using DNAStar, a program that searches for a specific gene sequence within another gene sequence. The results found the actinomycin D – DNA binding sequence is within the human CXCL10 DNA sequence (data not shown); therefore, indicating actinomycin D can bind to the specific gene of interest. The time-dependent inhibition of cytokine-induced CXCL10 mRNA transcripts by actinomycin D observed in the current study were similarly demonstrated in other studies (Dhillon et al., 2007; Galbis-Martinez et al., 2010; Okamoto et al., 2008; Shanmugam et al., 2006). For instance, CXCL10 mRNA transcript levels from IFNγ +/- PDGF (platelet-derived growth factor) stimulated monocytes were significantly decreased as early as 20 minutes post-actinomycin D treatment (Dhillon et al., 2007). The decrease of CXCL10 mRNA levels in the presence of actinomycin D continued to be significant even at the latest time point tested, 2 hours. A similar trend of CXCL10 mRNA transcript inhibition by actinomycin D occurred in murine embryonic fibroblasts stimulated with poly(ADP-ribose) polymerase-1 (Galbis-Martinez et al., 2010). Inhibition was present at 1 hour post-inhibitor exposure and continued for 12 hours. Therefore, the DNA-dependent RNA synthesis inhibitor actinomycin D is capable of blocking synthesis of new CXCL10 mRNA transcripts in A172 cells as well as other cell types.

Next, the effect of α-synuclein was examined on IL-1β-induced CXCL10 mRNA stability in A172 astroglial cells using an actinomycin D concentration of 10 µg/ml, which is a concentration consistent with that used by others (Gardner et al., 2006; Kasahara et al., 1991; Yao et al., 2005). The reduction of CXCL10 mRNA decay by α-synuclein relative to IL-1β treatment in the current study indicates that α-synuclein facilitates the stability of this CXCL10 mRNA in A172 cells. However, it remains unclear whether the effect by α-synuclein is direct or indirect. This is the first report on the role of α-synuclein on CXCL10 mRNA stability in IL-1β-exposed A172 cells. Interestingly, a single study has been reported regarding the role of α-synuclein on mRNA stability (Jin et al., 2011). This recent study by Jin et al. (2011) reported α-synuclein stably transfected into dopaminergic neurons resulted in protein kinase C-γ transcription suppression without any changes in mRNA stability. The effect by α-synuclein may be relative to this gene of interest in neuronal cells; however, it does not implicate a similar effect on other genes in different cell types. Furthermore, CXCL10 transcribed in the cell is expected to be degraded rapidly because of endogenous destabilizing mechanisms (Shanmugam et al., 2006). From the current data, results may suggest that α-synuclein may promote CXCL10 mRNA stability by delaying/inhibiting its degradation or destabilization. Certain proteins interact with mRNA transcripts to regulate mRNA degradation. From the list of these proteins (i.e. parp-1, HuR, S100b, nucleolin), only nucleolin has been shown to interact with α-synuclein (Jin et al., 2007). The apparent role nucleolin plays on stabilizing mRNA involves its participation with the 5’ and more so 3’ untranslated regions (5’ , 3’-UTR) of mRNA. Specifically, nucleolin binds to the 5’ and 3’ UTRs of specific mRNAs, enhancing both their stability and

translational efficiency. Jin et al. (2007) demonstrated the presence of nucleolin-bound α-synuclein through confocal microscopy, western immunoblotting, and immunoprecipitation (Jin et al., 2007). Confocal microscopy further revealed localization of nucleolin in both the nucleolus and cytoplasm compartments. In correlation with the current study, three possibilities for the increased mRNA stability by α-synuclein may be reasonable. One, aggregated α-synuclein may lead to a dissociation of nucleolin from α-synuclein and ultimately bind CXCL10 mRNA transcripts to promote stability. Secondly, a possibility may be the direct interaction of α-synuclein on stability. Alpha-synuclein, along with the bound nucleolin, may directly bind to the CXCL10 mRNA transcripts and enhance transcript stability. Jin et al. (2007) also demonstrated a significant reduction in nucleolin from α-synuclein in rat dopaminergic mesencephalic neuronal cell cultures after rotenone (a pesticide thought to be involved in PD) exposure. The nucleolin reduction may support the first possibility of dissociated nucleolin, though this may be a specific cause of rotenone and may differ with the current model. Third, α-synuclein may yet induce other stabilizing RNA-binding proteins or inhibit de-stabilizing RNA-binding proteins directly or indirectly, ultimately mediating CXCL10 mRNA stability.

## 5.4 Alpha-synuclein modifications on NF-κB signaling in cytokine-stimulated human A172 astroglial cells

The NF-κB signaling pathway is responsible for the induction of several inflammatory factors. The activation of NF-κB is examined by the translocation of NF-κB p65/p50 from the cytoplasm into the nucleus. In the current study, the significant up-regulation of the levels of active nuclear p65 protein by cytokine-alone correlates with other published data (Davis et al., 2007; Nadjar et al., 2003; Qi et al., 2009). For

instance, the exposure of A172 cells to TNFα resulted in an increase in nuclear p65 proteins (Davis et al., 2007). Other studies have demonstrated the ability of α-synuclein-alone to enhance NF-κB activation (Alberio et al., 2010; Klegeris et al., 2006; Reynolds et al., 2008b; Togo et al., 2001; Yuan et al., 2008). For instance, Reynolds et al. (2008) demonstrated an enhancement of p65 and p50 levels in the nucleus of microglial cells in the presence of aggregated and nitrated α-synuclein. The results by Reynold et al. (2008) were a measurement of total protein as oppose to active protein in the nucleus. Furthermore, the use of a higher concentration of α-synuclein as well as different cell-specific and α-synuclein preparation may have led to differences in results between the previous study and the current results. Klegeris et al. (2006) detected the activation of ERK1/2 and JNK MAPKs by non-aggregated α-synuclein in microglial cells, thus illustrating the involvement of other signaling pathways in glial cells by α-synuclein. Although several differences exist between Klegeris et al. and the present study (α-synuclein preparation and cell type), the outcomes do not eliminate the involvement of α-synuclein in NF-κB signaling due to the possible interactions between different signaling pathways. Nonetheless, the present results are the first report on α-synuclein modulation of levels of p65/p50 in A172 astroglial cells.

The lack of modulation observed by α-synuclein on IL-1β-induced phospho-IκBα and p65 levels was contrary to the observed enhancement of TNFα-induced p65 levels by α-synuclein. The results indicate a role of α-synuclein on NF-κB activation in the presence of TNFα. Although these results in the current study are the first to demonstrate α-synuclein's role on TNFα- and IL-1β-induced NF-κB activity, several lines of evidence support α-synuclein's involvement in inflammatory-induced NF-κB activity (Alberio et

al., 2010; Lee et al., 2010a; Prabhakaran et al., 2011; Wilms et al., 2009). For example, Parbhakaran et al. (2011) evaluated the mechanism involved in manganese-induced neurotoxicity in the presence of α-synuclein. Human α-synuclein enhanced manganese-induced neurotoxic susceptibility by activating the NF-κB signaling pathway, specifically NF-κB nuclear translocation. This enhancement was further blocked by an NF-κB inhibitor (SN50). The reason for the different results between TNFα and IL-1β-induced NF-κB activity by α-synuclein in the current study remains unclear. One explanation for the difference may be α-synuclein's ability to facilitate actions of the TNF receptor and/or the receptor's adaptor proteins, ultimately enhancing active p65 levels in the nucleus. Other explanations may be an activation of a different signaling pathway by α-synuclein in IL-1β-stimulated astrocytes. This latter explanation is in agreement with current results that demonstrate a lack of regulation by α-synuclein on the phosphorylation of IκBα, as well as with previous reports where α-synuclein activates other signaling pathways beside NF-κB, such as MAP kinase (Klegeris et al., 2008; Wilms et al., 2009). Astrocytes co-exposed to α-synuclein and IFNγ resulted in an enhanced activation of MAP kinase pathways p38, ERK1/2, and JNK via protein phosphorylation (Klegeris et al., 2008). Another explanation for the difference between α-synuclein's effect on TNF- and IL-1β-induced NF-κB activation may be due to cytokine concentrations used. The concentration of TNFα used in the current study may have been at a threshold level that allows α-synuclein to facilitate NF-κB activation, whereas IL-1β concentrations were not and therefore did not allow modulation by α-synuclein.

Interestingly, the lack of modulation on IL-1β-induced NF-κB by α-synuclein does not mirror the enhanced IL-1β-induced CXCL10 protein expression. In this case, α-synuclein may induce other signaling pathways in astrocytes that will mirror the induction of CXCL10 protein expression.

## 5.5 Alpha-synuclein did not alter cell viability

A number of *in vivo* and *in vitro* studies have reported the toxic actions of α-synuclein on oligodendroglial and neuronal cells (Bisaglia et al., 2010; Gao et al., 2011; Gu et al., 2010; Kragh et al., 2009; Zhang et al., 2005). For instance, Kragh et al. (2009) observations revealed a cellular degeneration and apoptosis in oligodendrocytes induced by α-synuclein. Additionally, aggregated α-synuclein at concentrations similar to the concentrations used in the current study resulted in dopaminergic neurotoxicity in rat primary mesencephalic neuronal cultures (Zhang et al., 2005). Neurotoxicity was even displayed in *in vivo* studies where α-synuclein resulted in significant neuronal loss in the midbrain region of mice models (Gao et al., 2011; Gu et al., 2010). Although these studies on neuronal cells illustrate a neurotoxic role of α-synuclein, A172 astroglial cells appear to be less susceptible to α-synuclein-induced cytotoxicity. This observation was the first report on α-synuclein's effect on A172 viability. Although concentrations of α-synuclein used in the current study did not impact astroglial viability, higher concentrations of this protein may ultimately result in a significant increase in cellular toxicity. Measures of cell viability, such as trypan blue and lactate dehydrogenase release assays, may give different results and therefore demonstrate fewer viable cells. Furthermore, astrocytes produce protective factors that result in an autocrine or paracrine effect, eventually demonstrating less susceptibility to toxic compounds. For instance, α-

synuclein is shown to induce oxidative stress in a number of cells, including astrocytes (Farooqui and Farooqui, 2011; McGeer and McGeer, 2008). Astrocytes, which produce the anti-oxidant glutathione under stressful conditions, may relieve the oxidative stress produced within the cell or in adjacent cells (Fuller 2009). Interestingly, the ability of α-synuclein to induce glutathione in response to heightened oxidative stress has been demonstrated (Hsu 2000). The possible explanations listed could result in less susceptibility of astrocytic data in α-synuclein pathology, yet further examination is needed.

## 5.6 Alpha-synuclein did not modulate cytokine-induced CXCL10 protein expression in Normal Human Astrocytes

In addition to examining the effects of α-synuclein on CXCL10 protein expression in human A172 astrocytoma cells, we concurrently examined these effects in normal human astrocytes (NHA). While IL-1β-stimulated CXCL10 levels were not affected by the current α-synuclein concentrations, other α-synuclein and IL-1β concentrations may lead to enhanced chemokine levels in NHA cells. For instance, CXCL10 protein levels may be modulated by higher α-synuclein and IL-1β concentrations than used in the current study. To date, only two groups of researchers have examined cytoactive molecules in α-synuclein-exposed primary astroglial cells. One group demonstrated a dose-dependent up-regulation of matrix metalloproteinase-9 by α-synuclein while observing a decrease of activity of the tissue plasminogen activator (Joo et al., 2010), whereas the other group demonstrated an increase in ICAM-1 and IL-6 (Klegeris et al., 2006). Therefore, although α-synuclein is shown to regulate certain molecules at the same concentrations that were used in the present study, this protein did

not result in a significant modification of the CXCL10 protein levels in the NHA cell line. Possible explanations for the lack of regulation by α-synuclein on NHA may be due to concentrations used or resistance to current α-synuclein configuration. Relative to NHA, A172 cells may be more sensitive to the effects of α-synuclein.

## 5.7 Alpha-synuclein elevated nitrite production in cytokine-stimulated human A172 astroglial cells

The inducible NOS isoform has received considerable attention due to the enzyme's ability to produce cytotoxic levels of NO. The up-regulation of iNOS, which is measured via nitrite accumulation, by a mixture of cytokines is supported by Davis et al. (2007), where an increase in iNOS activation is evident by astroglial NOS2 mRNA enhancement by the same cytokine mixture used in the current study. This study is the first to show α-synuclein mediated increases in cytokine-induced iNOS expression in human astroglial cells. Similarly, Gao et al. (2008) observed a nitrite accumulation and an increase in iNOS levels in microglial cells in the presence of α-synuclein with LPS relative to unstimulated cells. The study also revealed that the abatement of NO from α-synuclein and LPS co-exposed microglial cells provided significant neuroprotection in neuron-glia cultures. Another study revealed similar results, with an increase in microglial iNOS expression, which ultimately led to dopaminergic neuronal degeneration (Stefanova et al., 2007). The mechanism of α-synuclein mediated increase in iNOS expression is unclear, but may be a consequence of potentiating the activation of signaling pathways. For instance, an inhibition in glial iNOS expression in an *in vitro* MPTP model was mirrored with an inhibition of IκBα induction and p65 translocation into the nucleus, suggesting a role for NF-κB involvement in glial iNOS induction

(Dehmer et al., 2004). On the other hand, Waak and colleagues (2009) demonstrated an involvement of the p38 MAP kinase in the production of NO in DJ-1 mutated astrocytes (Waak et al., 2009). The results demonstrate the potential involvement of inflammatory signaling pathways that mediate the enhanced nitrite production by astrocytes.

The consequences of altered nitrite production in PD-associated neuropathology have not been fully elucidated. Depending on the temporal, regional, and magnitude of expression in the brain, iNOS expression may be neuroprotective or neurotoxic. At low levels, NO functions beneficially as both an antitumor and antimicrobial agent, whereas high levels of NO can be cytotoxic (Kroncke et al., 1997; Lipton, 1999; Liu and Stamler, 1999). In PD studies, an increase in iNOS expression and nitrite production results in neuronal death (Dehmer et al., 2004; Stefanova et al., 2007). Findings demonstrated an up-regulation of glial iNOS expression by α-synuclein and MPTP correlated with dopaminergic neuronal loss, with iNOS suppression protecting dopaminergic SNpc neurons (Dehmer et al., 2004; Stefanova et al., 2007). Although further examination is needed to elucidate the effect of α-synuclein-induced nitrite production in astrocytes, the current data suggest that NO released by astroglial cells may be a mediator that links neuroinflammation and aggregated α-synuclein in PD neurodegeneration.

# Part II

## Neuromelanin and oxidized dopamine modulation on inflammatory signaling in TNFα-stimulated human A172 astroglial cells

### 5.8 Neuromelanin and oxDA inhibit CXCL10 protein expression in A172 astroglial cells

Several studies have demonstrated that chemokines and chemokine receptors are associated with dopaminergic neurodegeneration (Rentzos et al., 2007; Shimoji et al., 2009) as evidenced by their increased expression. The consequences of altered chemokine (i.e, CXCL10) expression in dopaminergic associated neuropathology have not been fully elucidated. Modulation of astroglial CXCL10 expression by NM, could potentially have important implications in neuropathologies associated with dopaminergic neurons.

Our results indicate TNFα-induced CXCL10 expression in A172 astroglia is markedly decreased by oxDA and NM. This down-regulation of CXCL10 was not a consequence of altered cell viability by oxDA and NM in A172 cells; although, astroglial toxicity was seen when exposed to oxDA at the highest concentration used. There are no other reports in the literature describing the specific effects of oxDA or NM on CXCL10 expression in human astroglia. Although nothing is known about the effects of oxDA or NM on CXCL10 levels in PD, the reduction in the level of CXCL10 presented in our study provides new insights to the effects of oxDA or NM on proinflammatory-induced

CXCL10 expression in astroglia. Yet, it could be speculated that astroglial-derived CXCL10 plays a neuroprotective role in PD and therefore, oxDA or NM in excess could restrict this process, but further investigation is necessary. Another consideration of the effects of oxDA and NM is that astroglial CXCL10 expression may promote neurotoxicity and oxDA/NM reduction of the chemokine reflects a potential neuroprotective effect of NM. The idea that NM is neuroprotective is consistent with previous reports (Zecca et al., 2006). In fact, in physiological conditions the NM synthesis is a protective process which removes excess cytosolic DA which would otherwise cause neurotoxicity (Sulzer et al., 2000). Moreover, NM is a strong chelator of metals which blocks reactive toxic metals and forms stable complexes (Zecca et al., 2003). Once these actions of NM have been overwhelmed, the pathogenic actions of NM destroy NM-containing neurons, resulting in leakage of NM granules into the extracellular environment, and eventual disease aggravation.

### 5.9 Neuromelanin and oxDA reduce proinflammatory-induced active NF-κB signaling proteins in A172 astroglial cells

Activation of the transcription factor, NF-κB, is associated with dopaminergic associated neuropathology. For instance, within the ventral midbrain of PD patients, NF-κB p65 levels are elevated in microglia and astrocytes as compared to age-matched controls (Ghosh et al., 2009). Similarly, in a mouse model of PD, MPTP significantly induced NF-κB activation in astrocytes of the substantia nigra (Aoki et al., 2009). Furthermore, a previous study demonstrated that NF-κB activation is instrumental in TNFα-induced CXCL10 expression in human astroglial cells through the use of specific NF-κB inhibitors (Davis et al., 2007). In the current study, both oxDA and NM reduced

TNFα-induced NF-κB activation by decreasing levels of active nuclear p65 protein. The mechanism by which NM reduces NF-κB activation is unclear; however, several possibilities exist. Neuromelanin may influence the inhibition of IκBα phosphorylation or the exposure of nuclear translocation sequence on NF-κB proteins, thus reducing the amount of NF-κB proteins translocating into the nucleus. Neuromelanin may enter the nucleus, ultimately reducing the levels of active NF-κB proteins detected by binding to NF-κB proteins or facilitating the export of the proteins out of the nucleus. Additionally, the current results suggest that NM inhibits TNFα-induced CXCL10 expression in human astroglial cells, in part, through an NF-κB p65 dependent mechanism. The importance of NM mediated inhibition of NF-κB activation likely extends beyond just inhibition of CXCL10 expression. That is, the expression of many inflammatory molecules in astrocytes is transcriptionally regulated by NF-κB. Therefore, NM may inhibit transcription of multiple inflammatory molecules in astroglia. In contrast, in rat microglia, NM induces inflammatory signaling including NF-κB activation, and expression of inducible nitric oxide synthase and TNFα (Wilms et al., 2003; Zhang et al., 2011). Together, these findings suggest that NM modulation of inflammatory signaling may differ among glial cell types. However, this is the first report to assess the specific effects of oxDA or NM on astroglial NF-κB activation. This decline of NF-κB activation by oxDA or NM was parallel to a decline in both CXCL10 protein levels, proposing a possible correlation.

### 5.10 Oxidized dopamine- and neuromelanin-induced cytotoxicity in human neuronal and/or glial cells.

Previous studies suggest that NM-induced neurotoxicity is promoted by NM release from damaged dopaminergic neurons resulting in microglial activation and

production of neurotoxic molecules which further damage dopaminergic neurons (Wilms et al., 2003; Zecca et al., 2003; Zecca et al., 2006; Zecca et al., 2008; Zucca et al., 2004). In the current study, chemokine expression was not regulated by oxDA and NM alone; consequently, the effects of oxDA and NM on neuronal and glial viability were not of interest. In the presence of cytokines, a chronic exposure of SK-N-SH neuronal cells to oxDA results in a rapid reduction of viability in cells. Furthermore, chronic NM exposure dose-dependently reduces viability in TNFα-exposed SK-N-SH neuronal cultures. The oxDA- and NM-induced cytotoxicity in human neuronal cells may partly explain the neuronal toxicity observed in PD. For instance, others have shown that dopamine triggered apoptotic pathways in striatal neurons in *in vitro* and *in vivo* (McLaughlin et al., 1998) and induced oxidative stress and selective neurodegeneration in cell cultures (Stokes et al., 2000). Moreover, an oxidative-deamination metabolite of dopamine, 3,4-Dihydroxyphenylacetaldehyde (DOPAL) was responsible for neurodegeneration of PC12 cell cultures, possibly by inhibition of mitochondria complex I dysfunction (Lamensdorf et al., 2000).

Although previous works illustrate neurodegeneration by NM to be mediated by microglial cells (Rao et al., 2006; Wilms et al., 2003; Zecca et al., 2008), the current results show that NM exposed to dopaminergic SK-N-SH neuronal cells induces damage. In support of the NM-induced neurodegeneration, NM is shown to cause abnormal protein accumulation by inhibiting the proteolytic activities of the 26S proteasome and eventually lead to neuronal death in PD (Lopez Salon et al., 2003; Rao et al., 2006; Shamoto-Nagai et al., 2004; Song and Jung, 2004), or may selectively induce apoptosis in dopaminergic SH-SY5Y neuronal cells via mitochondrial deglutathionylation (Naoi et

al., 2008). In another study, NM (1-100 μg/ml) dose-dependently inhibited viability in primary cultures of mesencephalic dopaminergic neuronal cells (Nguyen et al., 2002). However, in this study, a synthetic melanin was employed and this has structure/behavior differences from human NM (Zecca et al., 2000). Compared to SK-N-SH neuronal cells, CHME-5 microglia and A172 astroglia are far less susceptible to oxDA- and NM-induced cytotoxicity. While previous studies concerning NM effects on microglia morphology and activation were performed with primary rat cultures (Wilms et al., 2003), this is the first report describing the effects of oxDA and NM on human microglial and astroglial viability. These findings are consistent with the concept that glial cells in the brain can survive and may even proliferate under conditions where neuronal cells degenerate (Hirsch et al., 2003; Wilms et al., 2003). Cellular cultures of primary neuronal and glia from PD *in vivo* rats cause mitigation of the neurodegenerative effects of oxDA and NM (Depboylu et al., 2007), in part through up-regulation of anti-inflammatory molecules or abatement of pro-inflammatory molecules and ROS production. The detrimental role of these activated glial cells may be associated with an up-regulation of proinflammatory molecules, including NO, cytokines and chemokines. In fact, several studies have demonstrated that dopamine and NM are inflammation-inducing molecules (Armentero et al., 2006; Hunot and Hirsch, 2003; Wilms et al., 2003; Zecca et al., 2006; Zecca et al., 2008). Therefore, while reactive microglia and reactive astrocytes are prominent in affected areas of the SN, neurodegeneration could occur due to a greater sensitivity of dopaminergic neurons to oxidative stress compared with glial cell sensitivity (Iwata-Ichikawa et al., 1999; McGeer and McGeer, 2008). This vulnerability to oxidative stress in dopaminergic neurons is caused by numerous factors.

Some examples include high amounts of dopamine molecules undergoing oxidation, low antioxidant protection due to low glutathione, high levels of lipid peroxide and iron deposition, and saturation of NM leading to increase levels of ROS (Double et al., 2002; Zecca et al., 2006; Zecca et al., 2008).

## 5.11 Oxidized dopamine and neuromelanin enhances proinflammatory-induced nitrite production in human astroglial cells

As mentioned before, numerous studies have demonstrated the involvement of iNOS in neuropathology associated with Parkinson disease (Aquilano et al., 2008; Arimoto and Bing, 2003; Hunot et al., 1996; Iravani et al., 2002; Marchetti et al., 2005). A cocktail of three cytokines (TNFα, IL-1β, and IFNγ) is required to induce iNOS expression in A172 cells (Davis and Syapin, 2004a; Davis and Syapin, 2004b). From the current results, the induction of iNOS by the cytokine mixture was not affected by oxDA or NM. This may reflect an inability of oxDA or NM to modulate maximal induction of iNOS that occurs in the presence of this cytokine mixture. TNFα alone is not sufficient to induce iNOS in A172 cells. However, suboptimal iNOS induction by TNFα alone is potentiated by oxDA and NM. A possible mechanism for NM's effect on TNFα-induced iNOS expression is activation of the signaling pathway. A number of studies have found the NF-κB pathway to be involved in iNOS expression (Nomura, 2001; Wang et al., 1999), however results from current study revealed an inhibition of NF-κB activation by NM. Other signaling pathways, such as the MAP kinase pathways, are also involved in the induction of iNOS expression (Kaminska, 2005) and the p38 MAP kinase, in particular, has been observed to be activated by human NM in glial cells (Wilms et al., 2003). Therefore, the induction of nitrite production by NM in TNFα-exposed astrocytes

may be due to activation of the p38 MAP kinase signaling pathway by NM in these cells, however, further examination is needed. Overall, the current study is the first to assess the specific effects of oxDA or NM on astroglial iNOS induction. Inducible NOS is upregulated in PD (Boka et al., 1994; Hirsch et al., 2003; Hunot et al., 1999; Knott et al., 2000) and; therefore, current results are in agreement with other glial studies (Rao et al., 2006; Wilms et al., 2003; Zecca et al., 2006; Zhang et al., 2011). For instance, extracellular NM was found to induce microglial production of NO (Wilms et al., 2003; Zecca et al., 2006; Zhang et al., 2011) as well as other inflammatory mediators. Nonetheless, this study provides novel insights into the effects of NM on inflammatory signaling in human astroglial cells. Depending on the temporal, regional, and magnitude of expression, astroglial release of NO may lead to a neuroprotective or neurotoxic effect. Although NO may be neuroprotective under certain conditions in the demylinating disease MS (Fenyk-Melody et al., 1998), PD studies demonstrate a more neurotoxic role of NO (Broom et al., 2011; Kim et al., 2010; Liberatore et al., 1999; Wilms et al., 2003). Consequently, a support of neurotoxic NO in PD may portray a significant effect on neurodegeneration by NM. Further analysis of the effects of NM on astroglial NO production may lead to an improved understanding of the roles and mechanisms of NM in PD.

# CHAPTER VI

## SUMMARY

This dissertation represents a two-hit model study that is designed to mimic the interactions between PD-associated proteins and inflammation in order to examine inflammatory signaling in human astroglial cells. This study was an investigation on the effects of two PD-associated molecules, α-synuclein and NM, on the modulation of proinflammatory signaling in human astroglial cells. Specifically, the objectives that were examined in the study were that (1) NM decresed while-synuclein increased cytokine-induced CXCL10 and iNOS expression and (2) both PD-associated proteins increased NF-κB activation in A172 astroglial cells.

The examination of inflammatory chemokine expression by α-synuclein agreed with a previous study that CXCL10 was up-regulated in PD model compared to healthy controls. Alpha-synuclein induced the expression of CXCL10 in both IL-1β- and TNFα-stimulated astroglial cells, however, this enhancement by α-synuclein was not demonstrated for the mRNA. Examining post-transcriptional regulation by α-synuclein on cytokine-induced CXCL10 mRNA revealed the ability of α-synuclein to mediate an increase in mRNA stability relative to cytokine-alone treatments. This regulation by α-synuclein may be, in part, the mechanism responsible for the increase in CXCL10 protein

expression by α-synuclein. The NF-κB signaling pathway was not activated by α-synuclein under similar treatments that induced CXCL10 expression. However, this pathway was activated in the presence of other α-synuclein concentrations in TNFα-stimulated cells, which may suggest the expression of other inflammatory mediators besides CXCL10. An increase in astroglial nitrite accumulation was also observed in the presence of α-synuclein, further supporting the inflammation-mediated effects by α-synuclein.

Investigation of role of NM on astroglial chemokine expression revealed a down-regulation of CXCL10 protein expression. This decrease in protein expression was mirrored with a reduction in NF-κB activation, suggesting a possible mechanism responsible for decrease of CXCL10 protein. On the other hand, NM enhanced cytokine-induced nitrite accumulation in the presence of astrocytes; however, further studies need to be conducted to understand the mechanism.

Indeed, aggregated α-synuclein and NM modulate astroglial CXCL10 chemokine production and iNOS induction. The changes in inflammatory chemokines and enzymes by α-synuclein and NM support a relation between inflammatory-mediated α-synuclein/NM alterations on astroglial cells. Although detailed mechanisms remain to be defined, these observations suggest a novel mechanism by which PD may progress. Together, these results suggest the pathogenic actions of NM and α-synuclein extend to the extracellular space and neighboring cells. Neuromelanin and α-synuclein proteins released from neurons may be important mediators of astroglial inflammatory responses in PD. Therefore, the consequences of α-synuclein and NM on astrocytes may cause a vicious self-propelling cycle that allows chronic neuroinflammation to persist long after

initiation. The effects of α-synuclein and NM observed in the present study provide novel information that may be useful in manipulating ongoing astroglial inflammatory responses. The use of anti-inflammatory drugs may therefore be beneficial and provide at least partial relief from neuroinflammation observed in PD.

In summary, these studies provide an initial understanding on the effects of PD-associated molecules and inflammation on human brain cells. Further increasing our knowledge of α-synuclein and NM mediated effects may provide new insights into the mechanism of disease progression and identify molecular targets for diagnosis and therapeutic intervention in PD.

| Treatments | Alpha-Synuclein | | | Neuromelanin | |
|---|---|---|---|---|---|
| | *+TNFα[@] + α-syn (11nM)* | *+(TNFα[$]+IL-1β*+IFNγ) + α-syn (300nM)* | *+IL-1β[#] + α-syn (33nM)* | *+TNFα[@] + NM (15μg/ml)* | *+TNFα[@] +NM (0.2-5μg/ml)* |
| **Results** | ↑CXCL10 protein expression | ↑iNOS expression | ↑CXCL10 protein expression | ↓CXCL10 protein expression | ↑iNOS expression |
| | No effect was observed on NF-κB signaling | | No effect was observed on NF-κB signaling and CXCL10 mRNA expression, yet ↑ in CXCL10 mRNA stability | Lower protein may be mediated in part by ↓ NF-κB signaling | |

*IFNγ (100 ng/ml)*
*[@] TNFα (5 ng/ml)*
*[$] TNFα (30 ng/ml)*
** IL-1β (5 ng/ml)*
*[#] IL-1β (0.25 ng/ml)*

**Table IX Overall summary.** A172 cells exposed to α-synuclein resulted in various outcomes, depending on α-synuclein and cytokine concentration. At the highest concentration examined, 300 nM α-synuclein and the cytokine mixture enhanced the expression of astroglial iNOS. At lower α-synuclein concentrations, CXCL10 protein expression was induced and may be mediated by an increase in mRNA stability in IL-1β-stimulated cells. On the other hand, neuromelanin (NM) decreased CXCL10 expression with a reduction of NF-κB signaling in TNFα-stimulated astroglial cell. Lower concentrations of NM resulted in an increase in iNOS expression in TNFα-stimulated cells.

Future studies on astrocytes would be conducted to elucidate the effects of α-synuclein and NM on : (1) TNFα-stimulated CXCL10 mRNA expression and NF-κB activation, (2) TNFα + IL-1β-induced CXCL10 expression, (3) iNOS mRNA expression and NF-κB activation (promoter studies), (4) endocytosis of α-synuclein and NM, (5) intracellular CXCL10 protein levels, (6) modulation of other chemokines and

ROS, (7) other various signaling pathways using specific inhibitors, and (8) neuronal degeneration via astroglial by-products, with degeneration blockage by natural or synthetic compounds.

## REFERENCES


Aggarwal BB, Moffat B and Harkins RN (1984) Human lymphotoxin. Production by a lymphoblastoid cell line, purification, and initial characterization. *J Biol Chem* 259:686-691.

Aggarwal BB, Eessalu TE and Hass PE (1985a) Characterization of receptors for human tumour necrosis factor and their regulation by gamma-interferon. *Nature* 318:665-667.

Aggarwal BB, Kohr WJ, Hass PE, Moffat B, Spencer SA, Henzel WJ, Bringman TS, Nedwin GE, Goeddel DV and Harkins RN (1985b) Human tumor necrosis factor. Production, purification, and characterization. *J Biol Chem* 260:2345-2354.

Alberio T, Bossi AM, Milli A, Parma E, Gariboldi MB, Tosi G, Lopiano L and Fasano M (2010) Proteomic analysis of dopamine and alpha-synuclein interplay in a cellular model of Parkinson's disease pathogenesis. *FEBS J* 277:4909-4919.

Allan SM, Tyrrell PJ and Rothwell NJ (2005) Interleukin-1 and neuronal injury. *Nat Rev Immunol* 5:629-640.

Anderson MF, Blomstrand F, Blomstrand C, Eriksson PS and Nilsson M (2003) Astrocytes and stroke: networking for survival? *Neurochem Res* 28:293-305.

Aoki E, Yano R, Yokoyama H, Kato H and Araki T (2009) Role of nuclear transcription factor kappa B (NF-kappaB) for MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahyropyridine)-induced apoptosis in nigral neurons of mice. *Exp Mol Pathol* 86:57-64.

Aquilano K, Baldelli S, Rotilio G and Ciriolo MR (2008) Role of nitric oxide synthases in Parkinson's disease: a review on the antioxidant and anti-inflammatory activity of polyphenols. *Neurochem Res* 33:2416-2426.

Arimoto T and Bing G (2003) Up-regulation of inducible nitric oxide synthase in the substantia nigra by lipopolysaccharide causes microglial activation and neurodegeneration. *Neurobiol Dis* 12:35-45.

Armentero MT, Levandis G, Nappi G, Bazzini E and Blandini F (2006) Peripheral inflammation and neuroprotection: systemic pretreatment with complete Freund's adjuvant reduces 6-hydroxydopamine toxicity in a rodent model of Parkinson's disease. *Neurobiol Dis* 24:492-505.

Aronson SM (1986) James Parkinson's shaking palsy. *R I Med J* 69:303-304.

Balkwill F (2009) Tumour necrosis factor and cancer. *Nat Rev Cancer* 9:361-371.

Barcia C, de Pablos V, Bautista-Hernandez V, Sanchez-Bahillo A, Bernal I, Fernandez-Villalba E, Martin J, Banon R, Fernandez-Barreiro A and Herrero MT (2005) Increased plasma levels of TNF-alpha but not of IL1-beta in MPTP-treated monkeys one year after the MPTP administration. *Parkinsonism Relat Disord* 11:435-439.

Barnum CJ, Eskow KL, Dupre K, Blandino P,Jr, Deak T and Bishop C (2008) Exogenous corticosterone reduces L-DOPA-induced dyskinesia in the hemi-parkinsonian rat: role for interleukin-1beta. *Neuroscience* 156:30-41.

Basu A, Krady JK and Levison SW (2004) Interleukin-1: a master regulator of neuroinflammation. *J Neurosci Res* 78:151-156.

Baud V and Karin M (2001) Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol* 11:372-377.

Bersani L, Colotta F and Mantovani A (1986) Involvement of tumour necrosis factor in monocyte-mediated rapid killing of actinomycin D-pretreated WEHI 164 sarcoma cells. *Immunology* 59:323-325.

Bisaglia M, Greggio E, Maric D, Miller DW, Cookson MR and Bubacco L (2010) Alpha-synuclein overexpression increases dopamine toxicity in BE2-M17 cells. *BMC Neurosci* 11:41.

Blasi F, Riccio M, Brogi A, Strazza M, Taddei ML, Romagnoli S, Luddi A, D'Angelo R, Santi S, Costantino-Ceccarini E and Melli M (1999) Constitutive expression of interleukin-1beta (IL-1beta) in rat oligodendrocytes. *Biol Chem* 380:259-264.

Blum-Degen D, Muller T, Kuhn W, Gerlach M, Przuntek H and Riederer P (1995) Interleukin-1 beta and interleukin-6 are elevated in the cerebrospinal fluid of Alzheimer's and de novo Parkinson's disease patients. *Neurosci Lett* 202:17-20.

Boka G, Anglade P, Wallach D, Javoy-Agid F, Agid Y and Hirsch EC (1994) Immunocytochemical analysis of tumor necrosis factor and its receptors in Parkinson's disease. *Neurosci Lett* 172:151-154.

Bonizzi G and Karin M (2004) The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol* 25:280-288.

Brambilla R, Bracchi-Ricard V, Hu WH, Frydel B, Bramwell A, Karmally S, Green EJ and Bethea JR (2005) Inhibition of astroglial nuclear factor kappaB reduces inflammation and improves functional recovery after spinal cord injury. *J Exp Med* 202:145-156.

Breder CD, Tsujimoto M, Terano Y, Scott DW and Saper CB (1993) Distribution and characterization of tumor necrosis factor-alpha-like immunoreactivity in the murine central nervous system. *J Comp Neurol* 337:543-567.

Bromberg-Martin ES, Matsumoto M and Hikosaka O (2010) Dopamine in motivational control: rewarding, aversive, and alerting. *Neuron* 68:815-834.

Broom L, Marinova-Mutafchieva L, Sadeghian M, Davis JB, Medhurst AD and Dexter DT (2011) Neuroprotection by the selective iNOS inhibitor GW274150 in a model of Parkinson disease. *Free Radic Biol Med* 50:633-640.

Carmichael J, DeGraff WG, Gazdar AF, Minna JD and Mitchell JB (1987) Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res* 47:936-942.

Carpentier PA, Begolka WS, Olson JK, Elhofy A, Karpus WJ and Miller SD (2005) Differential activation of astrocytes by innate and adaptive immune stimuli. *Glia* 49:360-374.

Carvey PM, Chen EY, Lipton JW, Tong CW, Chang QA and Ling ZD (2005) Intra-parenchymal injection of tumor necrosis factor-alpha and interleukin 1-beta produces dopamine neuron loss in the rat. *J Neural Transm* 112:601-612.

Chen H, Zhang SM, Hernan MA, Schwarzschild MA, Willett WC, Colditz GA, Speizer FE and Ascherio A (2003) Nonsteroidal anti-inflammatory drugs and the risk of Parkinson disease. *Arch Neurol* 60:1059-1064.

Chen H, O'Reilly EJ, Schwarzschild MA and Ascherio A (2008) Peripheral inflammatory biomarkers and risk of Parkinson's disease. *Am J Epidemiol* 167:90-95.

Chen PS, Peng GS, Li G, Yang S, Wu X, Wang CC, Wilson B, Lu RB, Gean PW, Chuang DM and Hong JS (2006) Valproate protects dopaminergic neurons in midbrain neuron/glia cultures by stimulating the release of neurotrophic factors from astrocytes. *Mol Psychiatry* 11:1116-1125.

Chen YG, Siddhanta A, Austin CD, Hammond SM, Sung TC, Frohman MA, Morris AJ and Shields D (1997) Phospholipase D stimulates release of nascent secretory vesicles from the trans-Golgi network. *J Cell Biol* 138:495-504.

Cicchetti F, Brownell AL, Williams K, Chen YI, Livni E and Isacson O (2002) Neuroinflammation of the nigrostriatal pathway during progressive 6-OHDA dopamine degeneration in rats monitored by immunohistochemistry and PET imaging. *Eur J Neurosci* 15:991-998.

Clayton DF and George JM (1998) The synucleins: a family of proteins involved in synaptic function, plasticity, neurodegeneration and disease. *Trends Neurosci* 21:249-254.

Cole AM, Ganz T, Liese AM, Burdick MD, Liu L and Strieter RM (2001) Cutting edge: IFN-inducible ELR- CXC chemokines display defensin-like antimicrobial activity. *J Immunol* 167:623-627.

Damier P, Hirsch EC, Zhang P, Agid Y and Javoy-Agid F (1993) Glutathione peroxidase, glial cells and Parkinson's disease. *Neuroscience* 52:1-6.

Dauer W and Przedborski S (2003) Parkinson's disease: mechanisms and models. *Neuron* 39:889-909.

Davies CA, Loddick SA, Toulmond S, Stroemer RP, Hunt J and Rothwell NJ (1999) The progression and topographic distribution of interleukin-1beta expression after permanent middle cerebral artery occlusion in the rat. *J Cereb Blood Flow Metab* 19:87-98.

Davis RL, Dertien J and Syapin PJ (2002) Ethanol-induced modulation of inducible nitric-oxide synthase activity in human A172 astrocytoma cells. *Alcohol Clin Exp Res* 26:1404-1411.

Davis RL and Syapin PJ (2004a) Acute ethanol exposure modulates expression of inducible nitric-oxide synthase in human astroglia: evidence for a transcriptional mechanism. *Alcohol* 32:195-202.

Davis RL and Syapin PJ (2004b) Chronic ethanol inhibits CXC chemokine ligand 10 production in human A172 astroglia and astroglial-mediated leukocyte chemotaxis. *Neurosci Lett* 362:220-225.

Davis RL, Sanchez AC, Lindley DJ, Williams SC and Syapin PJ (2005) Effects of mechanistically distinct NF-kappaB inhibitors on glial inducible nitric-oxide synthase expression. *Nitric Oxide* 12:200-209.

Davis RL, Buck DJ, Saffarian N and Stevens CW (2007) The opioid antagonist, beta-funaltrexamine, inhibits chemokine expression in human astroglial cells. *J Neuroimmunol* 186:141-149.

De Lella Ezcurra AL, Chertoff M, Ferrari C, Graciarena M and Pitossi F (2010) Chronic expression of low levels of tumor necrosis factor-alpha in the substantia nigra elicits progressive neurodegeneration, delayed motor symptoms and microglia/macrophage activation. *Neurobiol Dis* 37:630-640.

Dehmer T, Heneka MT, Sastre M, Dichgans J and Schulz JB (2004) Protection by pioglitazone in the MPTP model of Parkinson's disease correlates with I kappa B

alpha induction and block of NF kappa B and iNOS activation. *J Neurochem* 88:494-501.

Depboylu C, Matusch A, Tribl F, Zoriy M, Michel PP, Riederer P, Gerlach M, Becker S, Oertel WH and Hoglinger GU (2007) Glia protects neurons against extracellular human neuromelanin. *Neurodegener Dis* 4:218-226.

Derudder E, Dejardin E, Pritchard LL, Green DR, Korner M and Baud V (2003) RelB/p50 dimers are differentially regulated by tumor necrosis factor-alpha and lymphotoxin-beta receptor activation: critical roles for p100. *J Biol Chem* 278:23278-23284.

Deumens R, Blokland A and Prickaerts J (2002) Modeling Parkinson's disease in rats: an evaluation of 6-OHDA lesions of the nigrostriatal pathway. *Exp Neurol* 175:303-317.

Dev KK, Hofele K, Barbieri S, Buchman VL and van der Putten H (2003) Part II: alpha-synuclein and its molecular pathophysiological role in neurodegenerative disease. *Neuropharmacology* 45:14-44.

Dhillon NK, Peng F, Ransohoff RM and Buch S (2007) PDGF synergistically enhances IFN-gamma-induced expression of CXCL10 in blood-derived macrophages: implications for HIV dementia. *J Immunol* 179:2722-2730.

Dinarello CA(1988) Biology of interleukin 1. *FASEB J* 2:108-115.

Dinarello CA and Wolff SM (1993) The role of interleukin-1 in disease. *N Engl J Med* 328:106-113.

Dinarello CA(1996) Biologic basis for interleukin-1 in disease. *Blood* 87:2095-2147.

Dobbs RJ, Charlett A, Purkiss AG, Dobbs SM, Weller C and Peterson DW (1999) Association of circulating TNF-alpha and IL-6 with ageing and parkinsonism. *Acta Neurol Scand* 100:34-41.

Dopp JM, Mackenzie-Graham A, Otero GC and Merrill JE (1997) Differential expression, cytokine modulation, and specific functions of type-1 and type-2 tumor necrosis factor receptors in rat glia. *J Neuroimmunol* 75:104-112.

Double KL, Ben-Shachar D, Youdim MB, Zecca L, Riederer P and Gerlach M (2002) Influence of neuromelanin on oxidative pathways within the human substantia nigra. *Neurotoxicol Teratol* 24:621-628.

Dower SK, Kronheim SR, Hopp TP, Cantrell M, Deeley M, Gillis S, Henney CS and Urdal DL (1986) The cell surface receptors for interleukin-1 alpha and interleukin-1 beta are identical. *Nature* 324:266-268.

Dvoriantchikova G, Barakat D, Brambilla R, Agudelo C, Hernandez E, Bethea JR, Shestopalov VI and Ivanov D (2009) Inactivation of astroglial NF-kappa B promotes survival of retinal neurons following ischemic injury. *Eur J Neurosci* 30:175-185.

El-Agnaf OM, Jakes R, Curran MD, Middleton D, Ingenito R, Bianchi E, Pessi A, Neill D and Wallace A (1998) Aggregates from mutant and wild-type alpha-synuclein proteins and NAC peptide induce apoptotic cell death in human neuroblastoma cells by formation of beta-sheet and amyloid-like filaments. *FEBS Lett* 440:71-75.

El-Agnaf OM, Salem SA, Paleologou KE, Cooper LJ, Fullwood NJ, Gibson MJ, Curran MD, Court JA, Mann DM, Ikeda S, Cookson MR, Hardy J and Allsop D (2003) Alpha-synuclein implicated in Parkinson's disease is present in extracellular biological fluids, including human plasma. *FASEB J* 17:1945-1947.

El-Agnaf OM, Salem SA, Paleologou KE, Curran MD, Gibson MJ, Court JA, Schlossmacher MG and Allsop D (2006) Detection of oligomeric forms of alpha-synuclein protein in human plasma as a potential biomarker for Parkinson's disease. *FASEB J* 20:419-425.

Enochs WS, Sarna T, Zecca L, Riley PA and Swartz HM (1994) The roles of neuromelanin, binding of metal ions, and oxidative cytotoxicity in the pathogenesis of Parkinson's disease: a hypothesis. *J Neural Transm Park Dis Dement Sect* 7:83-100.

Ernandez T and Mayadas TN (2009) Immunoregulatory role of TNFalpha in inflammatory kidney diseases. *Kidney Int* 76:262-276.

Fahn S (2003) Description of Parkinson's disease as a clinical syndrome. *Ann N Y Acad Sci* 991:1-14.

Farooqui T and Farooqui AA (2011) Lipid-mediated oxidative stress and inflammation in the pathogenesis of Parkinson's disease. *Parkinsons Dis* 2011:247467.

Fedorow H, Tribl F, Halliday G, Gerlach M, Riederer P and Double KL (2005) Neuromelanin in human dopamine neurons: comparison with peripheral melanins and relevance to Parkinson's disease. *Prog Neurobiol* 75:109-124.

Fenyk-Melody JE, Garrison AE, Brunnert SR, Weidner JR, Shen F, Shelton BA and Mudgett JS (1998) Experimental autoimmune encephalomyelitis is exacerbated in mice lacking the NOS2 gene. *J Immunol* 160:2940-2946.

Ferger B, Leng A, Mura A, Hengerer B and Feldon J (2004) Genetic ablation of tumor necrosis factor-alpha (TNF-alpha) and pharmacological inhibition of TNF-synthesis attenuates MPTP toxicity in mouse striatum. *J Neurochem* 89:822-833.

Fernandes A, Falcao AS, Silva RF, Gordo AC, Gama MJ, Brito MA and Brites D (2006) Inflammatory signalling pathways involved in astroglial activation by unconjugated bilirubin. *J Neurochem* 96:1667-1679.

Ferrari CC, Pott Godoy MC, Tarelli R, Chertoff M, Depino AM and Pitossi FJ (2006) Progressive neurodegeneration and motor disabilities induced by chronic expression of IL-1beta in the substantia nigra. *Neurobiol Dis* 24:183-193.

Fields RD and Stevens-Graham B (2002) New insights into neuron-glia communication. *Science* 298:556-562.

Fillit H, Ding WH, Buee L, Kalman J, Altstiel L, Lawlor B and Wolf-Klein G (1991) Elevated circulating tumor necrosis factor levels in Alzheimer's disease. *Neurosci Lett* 129:318-320.

Flynn G, Maru S, Loughlin J, Romero IA and Male D (2003) Regulation of chemokine receptor expression in human microglia and astrocytes. *J Neuroimmunol* 136:84-93.

Forno LS, DeLanney LE, Irwin I, Di Monte D and Langston JW (1992) Astrocytes and Parkinson's disease. *Prog Brain Res* 94:429-436.

Fuller S, Munch G and Steele M (2009) Activated astrocytes: a therapeutic target in Alzheimer's disease? *Expert Rev Neurother* 9:1585-1594.

Furchgott RF and Zawadzki JV (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288:373-376.

Galbis-Martinez M, Saenz L, Ramirez P, Parrilla P and Yelamos J (2010) Poly(ADP-ribose) polymerase-1 modulates interferon-gamma-inducible protein (IP)-10 expression in murine embryonic fibroblasts by stabilizing IP-10 mRNA. *Mol Immunol* 47:1492-1499.

Galea E, Feinstein DL and Reis DJ (1992) Induction of calcium-independent nitric oxide synthase activity in primary rat glial cultures. *Proc Natl Acad Sci U S A* 89:10945-10949.

Gao HM, Kotzbauer PT, Uryu K, Leight S, Trojanowski JQ and Lee VM (2008) Neuroinflammation and oxidation/nitration of alpha-synuclein linked to dopaminergic neurodegeneration. *J Neurosci* 28:7687-7698.

Gao HM, Zhang F, Zhou H, Kam W, Wilson B and Hong JS (2011) Neuroinflammation and alpha-Synuclein Dysfunction Potentiate Each Other Driving Chronic Progression of Neurodegeneration in a Mouse Model of Parkinson's Disease. *Environ Health Perspect*

Gardner J, Borgmann K, Deshpande MS, Dhar A, Wu L, Persidsky R and Ghorpade A (2006) Potential mechanisms for astrocyte-TIMP-1 downregulation in chronic inflammatory diseases. *J Neurosci Res* 83:1281-1292.

George JM (2002) The synucleins. *Genome Biol* 3:REVIEWS3002.

Gerhard A, Pavese N, Hotton G, Turkheimer F, Es M, Hammers A, Eggert K, Oertel W, Banati RB and Brooks DJ (2006) In vivo imaging of microglial activation with [11C](R)-PK11195 PET in idiopathic Parkinson's disease. *Neurobiol Dis* 21:404-412.

Gery I, Gershon RK and Waksman BH (1972) Potentiation of the T-lymphocyte response to mitogens. I. The responding cell. *J Exp Med* 136:128-142.

Ghosh A, Roy A, Liu X, Kordower JH, Mufson EJ, Hartley DM, Ghosh S, Mosley RL, Gendelman HE and Pahan K (2007) Selective inhibition of NF-kappaB activation prevents dopaminergic neuronal loss in a mouse model of Parkinson's disease. *Proc Natl Acad Sci U S A* 104:18754-18759.

Ghosh A, Roy A, Matras J, Brahmachari S, Gendelman HE and Pahan K (2009) Simvastatin inhibits the activation of p21ras and prevents the loss of dopaminergic neurons in a mouse model of Parkinson's disease. *J Neurosci* 29:13543-13556.

Ghosh S and Karin M (2002) Missing pieces in the NF-kappaB puzzle. *Cell* 109 Suppl:S81-96.

Gibb WR and Lees AJ (1991) Anatomy, pigmentation, ventral and dorsal subpopulations of the substantia nigra, and differential cell death in Parkinson's disease. *J Neurol Neurosurg Psychiatry* 54:388-396.

Gibb WR(1992) Melanin, tyrosine hydroxylase, calbindin and substance P in the human midbrain and substantia nigra in relation to nigrostriatal projections and differential neuronal susceptibility in Parkinson's disease. *Brain Res* 581:283-291.

Grimaldi LM, Martino GV, Franciotta DM, Brustia R, Castagna A, Pristera R and Lazzarin A (1991) Elevated alpha-tumor necrosis factor levels in spinal fluid from HIV-1-infected patients with central nervous system involvement. *Ann Neurol* 29:21-25.

Gu XL, Long CX, Sun L, Xie C, Lin X and Cai H (2010) Astrocytic expression of Parkinson's disease-related A53T alpha-synuclein causes neurodegeneration in mice. *Mol Brain* 3:12.

Gupta SP, Patel S, Yadav S, Singh AK, Singh S and Singh MP (2010) Involvement of nitric oxide in maneb- and paraquat-induced Parkinson's disease phenotype in mouse: is there any link with lipid peroxidation? *Neurochem Res* 35:1206-1213.

Guthikonda P, Baker J and Mattson DH (1998) Interferon-beta-1-b (IFN-B) decreases induced nitric oxide (NO) production by a human astrocytoma cell line. *J Neuroimmunol* 82:133-139.

Hagan P, Barks JD, Yabut M, Davidson BL, Roessler B and Silverstein FS (1996) Adenovirus-mediated over-expression of interleukin-1 receptor antagonist reduces susceptibility to excitotoxic brain injury in perinatal rats. *Neuroscience* 75:1033-1045.

Hallegua DS and Weisman MH (2002) Potential therapeutic uses of interleukin 1 receptor antagonists in human diseases. *Ann Rheum Dis* 61:960-967.

Hannum CH, Wilcox CJ, Arend WP, Joslin FG, Dripps DJ, Heimdal PL, Armes LG, Sommer A, Eisenberg SP and Thompson RC (1990) Interleukin-1 receptor antagonist activity of a human interleukin-1 inhibitor. *Nature* 343:336-340.

Hastings TG, Lewis DA and Zigmond MJ (1996) Role of oxidation in the neurotoxic effects of intrastriatal dopamine injections. *Proc Natl Acad Sci U S A* 93:1956-1961.

Henning J, Strauss U, Wree A, Gimsa J, Rolfs A, Benecke R and Gimsa U (2008) Differential astroglial activation in 6-hydroxydopamine models of Parkinson's disease. *Neurosci Res* 62:246-253.

Hettinger AM, Allen MR, Zhang BR, Goad DW, Malayer JR and Geisert RD (2001) Presence of the acute phase protein, bikunin, in the endometrium of gilts during estrous cycle and early pregnancy. *Biol Reprod* 65:507-513.

Hirsch EC, Hunot S, Damier P and Faucheux B (1998) Glial cells and inflammation in Parkinson's disease: a role in neurodegeneration? *Ann Neurol* 44:S115-20.

Hirsch EC, Breidert T, Rousselet E, Hunot S, Hartmann A and Michel PP (2003) The role of glial reaction and inflammation in Parkinson's disease. *Ann N Y Acad Sci* 991:214-228.

Hirsch EC and Hunot S (2009) Neuroinflammation in Parkinson's disease: a target for neuroprotection? *Lancet Neurol* 8:382-397.

Hoehn MM and Yahr MD (1998) Parkinsonism: onset, progression, and mortality. 1967. *Neurology* 50:318.

Hofman FM, Hinton DR, Johnson K and Merrill JE (1989) Tumor necrosis factor identified in multiple sclerosis brain. *J Exp Med* 170:607-612.

Hsu LJ, Sagara Y, Arroyo A, Rockenstein E, Sisk A, Mallory M, Wong J, Takenouchi T, Hashimoto M and Masliah E (2000) Alpha-Synuclein Promotes Mitochondrial Deficit and Oxidative Stress. *Am J Pathol* 157:401-410.

Huang D, Pirskanen R, Hjelmstrom P and Lefvert AK (1998) Polymorphisms in IL-1beta and IL-1 receptor antagonist genes are associated with myasthenia gravis. *J Neuroimmunol* 81:76-81.

Hunot S, Boissiere F, Faucheux B, Brugg B, Mouatt-Prigent A, Agid Y and Hirsch EC (1996) Nitric oxide synthase and neuronal vulnerability in Parkinson's disease. *Neuroscience* 72:355-363.

Hunot S, Dugas N, Faucheux B, Hartmann A, Tardieu M, Debre P, Agid Y, Dugas B and Hirsch EC (1999) FcepsilonRII/CD23 is expressed in Parkinson's disease and induces, in vitro, production of nitric oxide and tumor necrosis factor-alpha in glial cells. *J Neurosci* 19:3440-3447.

Hunot S and Hirsch EC (2003) Neuroinflammatory processes in Parkinson's disease. *Ann Neurol* 53 Suppl 3:S49-58; discussion S58-60.

Iravani MM, Kashefi K, Mander P, Rose S and Jenner P (2002) Involvement of inducible nitric oxide synthase in inflammation-induced dopaminergic neurodegeneration. *Neuroscience* 110:49-58.

Ishikawa A and Takahashi H (1998) Clinical and neuropathological aspects of autosomal recessive juvenile parkinsonism. *J Neurol* 245:P4-9.

Iwai A, Masliah E, Yoshimoto M, Ge N, Flanagan L, de Silva HA, Kittel A and Saitoh T (1995) The precursor protein of non-A beta component of Alzheimer's disease amyloid is a presynaptic protein of the central nervous system. *Neuron* 14:467-475.

Iwata-Ichikawa E, Kondo Y, Miyazaki I, Asanuma M and Ogawa N (1999) Glial cells protect neurons against oxidative stress via transcriptional up-regulation of the glutathione synthesis. *J Neurochem* 72:2334-2344.

Janabi N, Peudenier S, Heron B, Ng KH and Tardieu M (1995) Establishment of human microglial cell lines after transfection of primary cultures of embryonic microglial cells with the SV40 large T antigen. *Neurosci Lett* 195:105-108.

Jin H, Kanthasamy A, Ghosh A, Yang Y, Anantharam V and Kanthasamy AG (2011) Alpha-synuclein negatively regulates protein kinase Cdelta expression to suppress apoptosis in dopaminergic neurons by reducing p300 histone acetyltransferase activity. *J Neurosci* 31:2035-2051.

Jin J, Li GJ, Davis J, Zhu D, Wang Y, Pan C and Zhang J (2007) Identification of novel proteins associated with both alpha-synuclein and DJ-1. *Mol Cell Proteomics* 6:845-859.

Jin T, Xu X and Hereld D (2008) Chemotaxis, chemokine receptors and human disease. *Cytokine* 44:1-8.

Jin ZB, Gu F, Ma X and Nao-i N (2007) Identification of a novel RPGR exon ORF15 mutation in a family with X-linked retinitis pigmentosa. *Arch Ophthalmol* 125:1407-1412.

John GR, Simpson JE, Woodroofe MN, Lee SC and Brosnan CF (2001) Extracellular nucleotides differentially regulate interleukin-1beta signaling in primary human astrocytes: implications for inflammatory gene expression. *J Neurosci* 21:4134-4142.

John GR, Lee SC, Song X, Rivieccio M and Brosnan CF (2005) IL-1-regulated responses in astrocytes: relevance to injury and recovery. *Glia* 49:161-176.

Joo SH, Kwon KJ, Kim JW, Kim JW, Hasan MR, Lee HJ, Han SH and Shin CY (2010) Regulation of matrix metalloproteinase-9 and tissue plasminogen activator activity by alpha-synuclein in rat primary glial cells. *Neurosci Lett* 469:352-356.

Kahle PJ, Neumann M, Ozmen L and Haass C (2000) Physiology and pathophysiology of alpha-synuclein. Cell culture and transgenic animal models based on a Parkinson's disease-associated protein. *Ann N Y Acad Sci* 920:33-41.

Kalkonde YV, Morgan WW, Sigala J, Maffi SK, Condello C, Kuziel W, Ahuja SS and Ahuja SK (2007) Chemokines in the MPTP model of Parkinson's disease: absence of CCL2 and its receptor CCR2 does not protect against striatal neurodegeneration. *Brain Res* 1128:1-11.

Kaminska B (2005) MAPK signalling pathways as molecular targets for anti-inflammatory therapy--from molecular mechanisms to therapeutic benefits. *Biochim Biophys Acta* 1754:253-262.

Kasahara T, Mukaida N, Yamashita K, Yagisawa H, Akahoshi T and Matsushima K (1991) IL-1 and TNF-alpha induction of IL-8 and monocyte chemotactic and activating factor (MCAF) mRNA expression in a human astrocytoma cell line. *Immunology* 74:60-67.

Kastner A, Hirsch EC, Lejeune O, Javoy-Agid F, Rascol O and Agid Y (1992) Is the vulnerability of neurons in the substantia nigra of patients with Parkinson's disease related to their neuromelanin content? *J Neurochem* 59:1080-1089.

Kim JS, Kim JM, O JJ and Jeon BS (2010) Inhibition of inducible nitric oxide synthase expression and cell death by (-)-epigallocatechin-3-gallate, a green tea catechin, in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. *J Clin Neurosci* 17:1165-1168.

Kirik D, Rosenblad C, Burger C, Lundberg C, Johansen TE, Muzyczka N, Mandel RJ and Bjorklund A (2002) Parkinson-like neurodegeneration induced by targeted

overexpression of alpha-synuclein in the nigrostriatal system. *J Neurosci* 22:2780-2791.

Klegeris A, Giasson BI, Zhang H, Maguire J, Pelech S and McGeer PL (2006) Alpha-synuclein and its disease-causing mutants induce ICAM-1 and IL-6 in human astrocytes and astrocytoma cells. *FASEB J* 20:2000-2008.

Klegeris A, Pelech S, Giasson BI, Maguire J, Zhang H, McGeer EG and McGeer PL (2008) Alpha-synuclein activates stress signaling protein kinases in THP-1 cells and microglia. *Neurobiol Aging* 29:739-752.

Klein RL, King MA, Hamby ME and Meyer EM (2002) Dopaminergic cell loss induced by human A30P alpha-synuclein gene transfer to the rat substantia nigra. *Hum Gene Ther* 13:605-612.

Knott C, Stern G and Wilkin GP (2000) Inflammatory regulators in Parkinson's disease: iNOS, lipocortin-1, and cyclooxygenases-1 and -2. *Mol Cell Neurosci* 16:724-739.

Koprich JB, Reske-Nielsen C, Mithal P and Isacson O (2008) Neuroinflammation mediated by IL-1beta increases susceptibility of dopamine neurons to degeneration in an animal model of Parkinson's disease. *J Neuroinflammation* 5:8.

Kragh CL, Lund LB, Febbraro F, Hansen HD, Gai WP, El-Agnaf O, Richter-Landsberg C and Jensen PH (2009) {alpha}-Synuclein Aggregation and Ser-129 Phosphorylation-dependent cell death in oligodendroglial cells. *J Biol Chem* 284:10211-10222.

Kriegler M, Perez C, DeFay K, Albert I and Lu SD (1988) A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF. *Cell* 53:45-53.

Kroncke KD, Fehsel K and Kolb-Bachofen V (1997) Nitric oxide: cytotoxicity versus cytoprotection--how, why, when, and where? *Nitric Oxide* 1:107-120.

Kubota N, Kiuchi Y, Nemoto M, Oyamada H, Ohno M, Funahashi H, Shioda S and Oguchi K (2001) Regulation of serotonin transporter gene expression in human glial cells by growth factors. *Eur J Pharmacol* 417:69-76.

Lamensdorf I, Eisenhofer G, Harvey-White J, Nechustan A, Kirk K and Kopin IJ (2000) 3,4-Dihydroxyphenylacetaldehyde potentiates the toxic effects of metabolic stress in PC12 cells. *Brain Res* 868:191-201.

Lang AE and Lozano AM (1998) Parkinson's disease. First of two parts. *N Engl J Med* 339:1044-1053.

Langston JW, Forno LS, Tetrud J, Reeves AG, Kaplan JA and Karluk D (1999) Evidence of active nerve cell degeneration in the substantia nigra of humans years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine exposure. *Ann Neurol* 46:598-605.

Lee EJ, Woo MS, Moon PG, Baek MC, Choi IY, Kim WK, Junn E and Kim HS (2010a) Alpha-synuclein activates microglia by inducing the expressions of matrix metalloproteinases and the subsequent activation of protease-activated receptor-1. *J Immunol* 185:615-623.

Lee HJ and Lee SJ (2002) Characterization of cytoplasmic alpha-synuclein aggregates. Fibril formation is tightly linked to the inclusion-forming process in cells. *J Biol Chem* 277:48976-48983.

Lee HJ, Suk JE, Patrick C, Bae EJ, Cho JH, Rho S, Hwang D, Masliah E and Lee SJ (2010b) Direct transfer of alpha-synuclein from neuron to astroglia causes inflammatory responses in synucleinopathies. *J Biol Chem* 285:9262-9272.

Lee M, Hyun D, Halliwell B and Jenner P (2001) Effect of the overexpression of wild-type or mutant alpha-synuclein on cell susceptibility to insult. *J Neurochem* 76:998-1009.

Lee SJ (2008) Origins and effects of extracellular alpha-synuclein: implications in Parkinson's disease. *J Mol Neurosci* 34:17-22.

Liberatore GT, Jackson-Lewis V, Vukosavic S, Mandir AS, Vila M, McAuliffe WG, Dawson VL, Dawson TM and Przedborski S (1999) Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson disease. *Nat Med* 5:1403-1409.

Lipton SA (1999) Neuronal protection and destruction by NO. *Cell Death Differ* 6:943-951.

Liu L and Stamler JS (1999) NO: an inhibitor of cell death. *Cell Death Differ* 6:937-942.

Long-Smith CM, Sullivan AM and Nolan YM (2009) The influence of microglia on the pathogenesis of Parkinson's disease. *Prog Neurobiol* 89:277-287.

Long-Smith CM, Collins L, Toulouse A, Sullivan AM and Nolan YM (2010) Interleukin-1beta contributes to dopaminergic neuronal death induced by lipopolysaccharide-stimulated rat glia in vitro. *J Neuroimmunol* 226:20-26.

Lopez Salon M, Pasquini L, Besio Moreno M, Pasquini JM and Soto E (2003) Relationship between beta-amyloid degradation and the 26S proteasome in neural cells. *Exp Neurol* 180:131-143.

Luster AD, Unkeless JC and Ravetch JV (1985) Gamma-interferon transcriptionally regulates an early-response gene containing homology to platelet proteins. *Nature* 315:672-676.

Luster AD and Ravetch JV (1987) Biochemical characterization of a gamma interferon-inducible cytokine (IP-10). *J Exp Med* 166:1084-1097.

Luster AD (1998) Chemokines--chemotactic cytokines that mediate inflammation. *N Engl J Med* 338:436-445.

Maier A, Zimmermann C, Beglinger C, Drewe J and Gutmann H (2007) Effects of budesonide on P-glycoprotein expression in intestinal cell lines. *Br J Pharmacol* 150:361-368.

Malek S, Chen Y, Huxford T and Ghosh G (2001) IkappaBbeta, but not IkappaBalpha, functions as a classical cytoplasmic inhibitor of NF-kappaB dimers by masking both NF-kappaB nuclear localization sequences in resting cells. *J Biol Chem* 276:45225-45235.

Mann DM and Yates PO (1983) Possible role of neuromelanin in the pathogenesis of Parkinson's disease. *Mech Ageing Dev* 21:193-203.

Maragakis NJ and Rothstein JD (2006) Mechanisms of Disease: astrocytes in neurodegenerative disease. *Nat Clin Pract Neurol* 2:679-689.

March CJ, Mosley B, Larsen A, Cerretti DP, Braedt G, Price V, Gillis S, Henney CS, Kronheim SR and Grabstein K (1985) Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. *Nature* 315:641-647.

Marchetti B, Serra PA, L'Episcopo F, Tirolo C, Caniglia S, Testa N, Cioni S, Gennuso F, Rocchitta G, Desole MS, Mazzarino MC, Miele E and Morale MC (2005) Hormones are key actors in gene x environment interactions programming the vulnerability to Parkinson's disease: glia as a common final pathway. *Ann N Y Acad Sci* 1057:296-318.

Maries E, Dass B, Collier TJ, Kordower JH and Steece-Collier K (2003) The role of alpha-synuclein in Parkinson's disease: insights from animal models. *Nat Rev Neurosci* 4:727-738.

Masliah E, Rockenstein E, Veinbergs I, Mallory M, Hashimoto M, Takeda A, Sagara Y, Sisk A and Mucke L (2000) Dopaminergic loss and inclusion body formation in alpha-synuclein mice: implications for neurodegenerative disorders. *Science* 287:1265-1269.

McCoy MK, Martinez TN, Ruhn KA, Szymkowski DE, Smith CG, Botterman BR, Tansey KE and Tansey MG (2006) Blocking soluble tumor necrosis factor signaling

with dominant-negative tumor necrosis factor inhibitor attenuates loss of dopaminergic neurons in models of Parkinson's disease. *J Neurosci* 26:9365-9375.

McFarland HF and Martin R (2007) Multiple sclerosis: a complicated picture of autoimmunity. *Nat Immunol* 8:913-919.

McGeer EG and McGeer PL (1998a) The importance of inflammatory mechanisms in Alzheimer disease. *Exp Gerontol* 33:371-378.

McGeer PL, Itagaki S, Boyes BE and McGeer EG (1988) Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. *Neurology* 38:1285-1291.

McGeer PL and McGeer EG (1998b) Glial cell reactions in neurodegenerative diseases: pathophysiology and therapeutic interventions. *Alzheimer Dis Assoc Disord* 12 Suppl 2:S1-6.

McGeer PL, Yasojima K and McGeer EG (2001) Inflammation in Parkinson's disease. *Adv Neurol* 86:83-89.

McGeer PL, Yasojima K and McGeer EG (2002) Association of interleukin-1 beta polymorphisms with idiopathic Parkinson's disease. *Neurosci Lett* 326:67-69.

McGeer PL and McGeer EG (2008) Glial reactions in Parkinson's disease. *Mov Disord* 23:474-483.

McGuire SO, Ling ZD, Lipton JW, Sortwell CE, Collier TJ and Carvey PM (2001) Tumor necrosis factor alpha is toxic to embryonic mesencephalic dopamine neurons. *Exp Neurol* 169:219-230.

McLaughlin BA, Nelson D, Erecinska M and Chesselet MF (1998) Toxicity of dopamine to striatal neurons in vitro and potentiation of cell death by a mitochondrial inhibitor. *J Neurochem* 70:2406-2415.

Mennini T, Bigini P, Cagnotto A, Carvelli L, Di Nunno P, Fumagalli E, Tortarolo M, Buurman WA, Ghezzi P and Bendotti C (2004) Glial activation and TNFR-I upregulation precedes motor dysfunction in the spinal cord of mnd mice. *Cytokine* 25:127-135.

Miklossy J, Doudet DD, Schwab C, Yu S, McGeer EG and McGeer PL (2006) Role of ICAM-1 in persisting inflammation in Parkinson disease and MPTP monkeys. *Exp Neurol* 197:275-283.

Miller JA, Trout BR, Sullivan KA, Bialecki RA, Roberts RA and Tjalkens RB (2011) Low-dose 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine causes inflammatory activation of astrocytes in nuclear factor-kappaB reporter mice prior to loss of dopaminergic neurons. *J Neurosci Res* 89:406-417.

Mizuno Y, Hattori N, Kubo S, Sato S, Nishioka K, Hatano T, Tomiyama H, Funayama M, Machida Y and Mochizuki H (2008) Progress in the pathogenesis and genetics of Parkinson's disease. *Philos Trans R Soc Lond B Biol Sci* 363:2215-2227.

Mogi M, Harada M, Kondo T, Riederer P, Inagaki H, Minami M and Nagatsu T (1994a) Interleukin-1 beta, interleukin-6, epidermal growth factor and transforming growth factor-alpha are elevated in the brain from parkinsonian patients. *Neurosci Lett* 180:147-150.

Mogi M, Harada M, Riederer P, Narabayashi H, Fujita K and Nagatsu T (1994b) Tumor necrosis factor-alpha (TNF-alpha) increases both in the brain and in the cerebrospinal fluid from parkinsonian patients. *Neurosci Lett* 165:208-210.

Mogi M, Togari A, Tanaka K, Ogawa N, Ichinose H and Nagatsu T (1999) Increase in level of tumor necrosis factor (TNF)-alpha in 6-hydroxydopamine-lesioned striatum in rats without influence of systemic L-DOPA on the TNF-alpha induction. *Neurosci Lett* 268:101-104.

Mogi M, Togari A, Kondo T, Mizuno Y, Komure O, Kuno S, Ichinose H and Nagatsu T (2000) Caspase activities and tumor necrosis factor receptor R1 (p55) level are elevated in the substantia nigra from parkinsonian brain. *J Neural Transm* 107:335-341.

Morale MC, Serra PA, L'episcopo F, Tirolo C, Caniglia S, Testa N, Gennuso F, Giaquinta G, Rocchitta G, Desole MS, Miele E and Marchetti B (2006) Estrogen, neuroinflammation and neuroprotection in Parkinson's disease: glia dictates resistance versus vulnerability to neurodegeneration. *Neuroscience* 138:869-878.

Mukhopadhyay A, Suttles J, Stout RD and Aggarwal BB (2001) Genetic deletion of the tumor necrosis factor receptor p60 or p80 abrogates ligand-mediated activation of nuclear factor-kappa B and of mitogen-activated protein kinases in macrophages. *J Biol Chem* 276:31906-31912.

Murphy DD, Rueter SM, Trojanowski JQ and Lee VM (2000) Synucleins are developmentally expressed, and alpha-synuclein regulates the size of the presynaptic vesicular pool in primary hippocampal neurons. *J Neurosci* 20:3214-3220.

Nadjar A, Combe C, Laye S, Tridon V, Dantzer R, Amedee T and Parnet P (2003) Nuclear factor kappaB nuclear translocation as a crucial marker of brain response to interleukin-1. A study in rat and interleukin-1 type I deficient mouse. *J Neurochem* 87:1024-1036.

Naoi M, Maruyama W, Yi H, Yamaoka Y, Shamoto-Nagai M, Akao Y, Gerlach M, Tanaka M and Riederer P (2008) Neuromelanin selectively induces apoptosis in dopaminergic SH-SY5Y cells by deglutathionylation in mitochondria: involvement of the protein and melanin component. *J Neurochem* 105:2489-2500.

Nguyen A, Gille G, Moldzio R, Hung ST and Rausch WD (2002) Synthetic neuromelanin is toxic to dopaminergic cell cultures. *J Neural Transm* 109:651-661.

Nicoll JA, Mrak RE, Graham DI, Stewart J, Wilcock G, MacGowan S, Esiri MM, Murray LS, Dewar D, Love S, Moss T and Griffin WS (2000) Association of interleukin-1 gene polymorphisms with Alzheimer's disease. *Ann Neurol* 47:365-368.

Niranjan R, Nath C and Shukla R (2010) The mechanism of action of MPTP-induced neuroinflammation and its modulation by melatonin in rat astrocytoma cells, C6. *Free Radic Res* 44:1304-1316.

Nishimura M, Mizuta I, Mizuta E, Yamasaki S, Ohta M, Kaji R and Kuno S (2001) Tumor necrosis factor gene polymorphisms in patients with sporadic Parkinson's disease. *Neurosci Lett* 311:1-4.

Nishimura M, Kuno S, Kaji R, Yasuno K and Kawakami H (2005) Glutathione-S-transferase-1 and interleukin-1beta gene polymorphisms in Japanese patients with Parkinson's disease. *Mov Disord* 20:901-902.

Nomura Y (2001) NF-kappaB activation and IkappaB alpha dynamism involved in iNOS and chemokine induction in astroglial cells. *Life Sci* 68:1695-1701.

Norris EH, Giasson BI and Lee VM (2004) Alpha-synuclein: normal function and role in neurodegenerative diseases. *Curr Top Dev Biol* 60:17-54.

Offen D, Ziv I, Barzilai A, Gorodin S, Glater E, Hochman A and Melamed E (1997) Dopamine-melanin induces apoptosis in PC12 cells; possible implications for the etiology of Parkinson's disease. *Neurochem Int* 31:207-216.

Oh JW, Schwiebert LM and Benveniste EN (1999) Cytokine regulation of CC and CXC chemokine expression by human astrocytes. *J Neurovirol* 5:82-94.

Ohmori Y and Hamilton TA (1995) The interferon-stimulated response element and a kappa B site mediate synergistic induction of murine IP-10 gene transcription by IFN-gamma and TNF-alpha. *J Immunol* 154:5235-5244.

Okamoto Y, Folco EJ, Minami M, Wara AK, Feinberg MW, Sukhova GK, Colvin RA, Kihara S, Funahashi T, Luster AD and Libby P (2008) Adiponectin inhibits the production of CXC receptor 3 chemokine ligands in macrophages and reduces T-lymphocyte recruitment in atherogenesis. *Circ Res* 102:218-225.

Onyango IG(2008) Mitochondrial dysfunction and oxidative stress in Parkinson's disease. *Neurochem Res* 33:589-597.

Padovan-Neto FE, Echeverry MB, Tumas V and Del-Bel EA (2009) Nitric oxide synthase inhibition attenuates L-DOPA-induced dyskinesias in a rodent model of Parkinson's disease. *Neuroscience* 159:927-935.

Pakkenberg B, Moller A, Gundersen HJ, Mouritzen Dam A and Pakkenberg H (1991) The absolute number of nerve cells in substantia nigra in normal subjects and in patients with Parkinson's disease estimated with an unbiased stereological method. *J Neurol Neurosurg Psychiatry* 54:30-33.

Palladino MA, Bahjat FR, Theodorakis EA and Moldawer LL (2003) Anti-TNF-alpha therapies: the next generation. *Nat Rev Drug Discov* 2:736-746.

Pearson VL, Rothwell NJ and Toulmond S (1999) Excitotoxic brain damage in the rat induces interleukin-1beta protein in microglia and astrocytes: correlation with the progression of cell death. *Glia* 25:311-323.

Perez RG, Waymire JC, Lin E, Liu JJ, Guo F and Zigmond MJ (2002) A role for alpha-synuclein in the regulation of dopamine biosynthesis. *J Neurosci* 22:3090-3099.

Pfeffer K (2003) Biological functions of tumor necrosis factor cytokines and their receptors. *Cytokine Growth Factor Rev* 14:185-191.

Prabhakaran K, Chapman GD and Gunasekar PG (2011) alpha-Synuclein overexpression enhances manganese-induced neurotoxicity through the NF-kappaB-mediated pathway. *Toxicol Mech Methods*

Qi XF, Kim DH, Yoon YS, Jin D, Huang XZ, Li JH, Deung YK and Lee KJ (2009) Essential involvement of cross-talk between IFN-gamma and TNF-alpha in CXCL10 production in human THP-1 monocytes. *J Cell Physiol* 220:690-697.

Rao KS, Hegde ML, Anitha S, Musicco M, Zucca FA, Turro NJ and Zecca L (2006) Amyloid beta and neuromelanin--toxic or protective molecules? The cellular context makes the difference. *Prog Neurobiol* 78:364-373.

Rentzos M, Nikolaou C, Andreadou E, Paraskevas GP, Rombos A, Zoga M, Tsoutsou A, Boufidou F, Kapaki E and Vassilopoulos D (2007) Circulating interleukin-15 and RANTES chemokine in Parkinson's disease. *Acta Neurol Scand* 116:374-379.

Reynolds AD, Glanzer JG, Kadiu I, Ricardo-Dukelow M, Chaudhuri A, Ciborowski P, Cerny R, Gelman B, Thomas MP, Mosley RL and Gendelman HE (2008a) Nitrated alpha-synuclein-activated microglial profiling for Parkinson's disease. *J Neurochem* 104:1504-1525.

Reynolds AD, Kadiu I, Garg SK, Glanzer JG, Nordgren T, Ciborowski P, Banerjee R and Gendelman HE (2008b) Nitrated alpha-synuclein and microglial neuroregulatory activities. *J Neuroimmune Pharmacol* 3:59-74.

Rodrigues RW, Gomide VC and Chadi G (2001) Astroglial and microglial reaction after a partial nigrostriatal degeneration induced by the striatal injection of different doses of 6-hydroxydopamine. *Int J Neurosci* 109:91-126.

Roodveldt C, Labrador-Garrido A, Gonzalez-Rey E, Fernandez-Montesinos R, Caro M, Lachaud CC, Waudby CA, Delgado M, Dobson CM and Pozo D (2010) Glial innate immunity generated by non-aggregated alpha-synuclein in mouse: differences between wild-type and Parkinson's disease-linked mutants. *PLoS One* 5:e13481.

Rothwell NJ and Luheshi GN (2000) Interleukin 1 in the brain: biology, pathology and therapeutic target. *Trends Neurosci* 23:618-625.

Rousselet E, Callebert J, Parain K, Joubert C, Hunot S, Hartmann A, Jacque C, Perez-Diaz F, Cohen-Salmon C, Launay JM and Hirsch EC (2002) Role of TNF-alpha receptors in mice intoxicated with the parkinsonian toxin MPTP. *Exp Neurol* 177:183-192.

Saavedra A, Baltazar G and Duarte EP (2007) Interleukin-1beta mediates GDNF up-regulation upon dopaminergic injury in ventral midbrain cell cultures. *Neurobiol Dis* 25:92-104.

Sairanen TR, Lindsberg PJ, Brenner M, Carpen O and Siren A (2001) Differential cellular expression of tumor necrosis factor-alpha and Type I tumor necrosis factor receptor after transient global forebrain ischemia. *J Neurol Sci* 186:87-99.

Sallusto F, Mackay CR and Lanzavecchia A (1997) Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. *Science* 277:2005-2007.

Savica R, Rocca WA and Ahlskog JE (2010) When does Parkinson disease start? *Arch Neurol* 67:798-801.

Savitt JM, Dawson VL and Dawson TM (2006) Diagnosis and treatment of Parkinson disease: molecules to medicine. *J Clin Invest* 116:1744-1754.

Sen R and Baltimore D (1986) Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. *Cell* 47:921-928.

Shamoto-Nagai M, Maruyama W, Akao Y, Osawa T, Tribl F, Gerlach M, Zucca FA, Zecca L, Riederer P and Naoi M (2004) Neuromelanin inhibits enzymatic activity of 26S proteasome in human dopaminergic SH-SY5Y cells. *J Neural Transm* 111:1253-1265.

Shanmugam N, Ransohoff RM and Natarajan R (2006) Interferon-gamma-inducible protein (IP)-10 mRNA stabilized by RNA-binding proteins in monocytes treated with S100b. *J Biol Chem* 281:31212-31221.

Sheng JG, Boop FA, Mrak RE and Griffin WS (1994) Increased neuronal beta-amyloid precursor protein expression in human temporal lobe epilepsy: association with interleukin-1 alpha immunoreactivity. *J Neurochem* 63:1872-1879.

Shimoji M, Pagan F, Healton EB and Mocchetti I (2009) CXCR4 and CXCL12 expression is increased in the nigro-striatal system of Parkinson's disease. *Neurotox Res* 16:318-328.

Sidhu A, Wersinger C and Vernier P (2004) Does alpha-synuclein modulate dopaminergic synaptic content and tone at the synapse? *FASEB J* 18:637-647.

Simi A, Tsakiri N, Wang P and Rothwell NJ (2007) Interleukin-1 and inflammatory neurodegeneration. *Biochem Soc Trans* 35:1122-1126.

Song S and Jung YK (2004) Alzheimer's disease meets the ubiquitin-proteasome system. *Trends Mol Med* 10:565-570.

Spillantini MG, Crowther RA, Jakes R, Hasegawa M and Goedert M (1998) alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. *Proc Natl Acad Sci U S A* 95:6469-6473.

Sriram K, Matheson JM, Benkovic SA, Miller DB, Luster MI and O'Callaghan JP (2002) Mice deficient in TNF receptors are protected against dopaminergic neurotoxicity: implications for Parkinson's disease. *FASEB J* 16:1474-1476.

Stefanova N, Reindl M, Neumann M, Kahle PJ, Poewe W and Wenning GK (2007) Microglial activation mediates neurodegeneration related to oligodendroglial alpha-synucleinopathy: implications for multiple system atrophy. *Mov Disord* 22:2196-2203.

Steinert JR, Chernova T and Forsythe ID (2010) Nitric oxide signaling in brain function, dysfunction, and dementia. *Neuroscientist* 16:435-452.

Stokes AH, Lewis DY, Lash LH, Jerome WG,3rd, Grant KW, Aschner M and Vrana KE (2000) Dopamine toxicity in neuroblastoma cells: role of glutathione depletion by L-BSO and apoptosis. *Brain Res* 858:1-8.

Su X, Federoff HJ and Maguire-Zeiss KA (2009) Mutant alpha-synuclein overexpression mediates early proinflammatory activity. *Neurotox Res* 16:238-254.

Su X, Maguire-Zeiss KA, Giuliano R, Prifti L, Venkatesh K and Federoff HJ (2008) Synuclein activates microglia in a model of Parkinson's disease. *Neurobiol Aging* 29:1690-1701.

Subramaniam S, Stansberg C and Cunningham C (2004) The interleukin 1 receptor family. *Dev Comp Immunol* 28:415-428.

Sui Y, Potula R, Dhillon N, Pinson D, Li S, Nath A, Anderson C, Turchan J, Kolson D, Narayan O and Buch S (2004) Neuronal apoptosis is mediated by CXCL10 overexpression in simian human immunodeficiency virus encephalitis. *Am J Pathol* 164:1557-1566.

Sui Y, Stehno-Bittel L, Li S, Loganathan R, Dhillon NK, Pinson D, Nath A, Kolson D, Narayan O and Buch S (2006) CXCL10-induced cell death in neurons: role of calcium dysregulation. *Eur J Neurosci* 23:957-964.

Sulzer D (2007) Multiple hit hypotheses for dopamine neuron loss in Parkinson's disease. *Trends Neurosci* 30:244-250.

Sulzer D, Bogulavsky J, Larsen KE, Behr G, Karatekin E, Kleinman MH, Turro N, Krantz D, Edwards RH, Greene LA and Zecca L (2000) Neuromelanin biosynthesis is driven by excess cytosolic catecholamines not accumulated by synaptic vesicles. *Proc Natl Acad Sci U S A* 97:11869-11874.

Sun D and Ding A (2006) MyD88-mediated stabilization of interferon-gamma-induced cytokine and chemokine mRNA. *Nat Immunol* 7:375-381.

Tansey MG, McCoy MK and Frank-Cannon TC (2007) Neuroinflammatory mechanisms in Parkinson's disease: potential environmental triggers, pathways, and targets for early therapeutic intervention. *Exp Neurol* 208:1-25.

Tanuma N, Sakuma H, Sasaki A and Matsumoto Y (2006) Chemokine expression by astrocytes plays a role in microglia/macrophage activation and subsequent neurodegeneration in secondary progressive multiple sclerosis. *Acta Neuropathol* 112:195-204.

Taub DD, Lloyd AR, Conlon K, Wang JM, Ortaldo JR, Harada A, Matsushima K, Kelvin DJ and Oppenheim JJ (1993) Recombinant human interferon-inducible protein 10 is a chemoattractant for human monocytes and T lymphocytes and promotes T cell adhesion to endothelial cells. *J Exp Med* 177:1809-1814.

Taylor BS, de Vera ME, Ganster RW, Wang Q, Shapiro RA, Morris SM,Jr, Billiar TR and Geller DA (1998) Multiple NF-kappaB enhancer elements regulate cytokine induction of the human inducible nitric oxide synthase gene. *J Biol Chem* 273:15148-15156.

Teismann P and Schulz JB (2004) Cellular pathology of Parkinson's disease: astrocytes, microglia and inflammation. *Cell Tissue Res* 318:149-161.

Tiveron MC, Rossel M, Moepps B, Zhang YL, Seidenfaden R, Favor J, Konig N and Cremer H (2006) Molecular interaction between projection neuron precursors and invading interneurons via stromal-derived factor 1 (CXCL12)/CXCR4 signaling in the cortical subventricular zone/intermediate zone. *J Neurosci* 26:13273-13278.

Togo T, Iseki E, Marui W, Akiyama H, Ueda K and Kosaka K (2001) Glial involvement in the degeneration process of Lewy body-bearing neurons and the degradation process of Lewy bodies in brains of dementia with Lewy bodies. *J Neurol Sci* 184:71-75.

Tower DB and Young OM (1973) The activities of butyrylcholinesterase and carbonic anhydrase, the rate of anaerobic glycolysis, and the question of a constant density of glial cells in cerebral cortices of various mammalian species from mouse to whale. *J Neurochem* 20:269-278.

Ubogu EE, Cossoy MB and Ransohoff RM (2006) The expression and function of chemokines involved in CNS inflammation. *Trends Pharmacol Sci* 27:48-55.

Ueda K, Fukushima H, Masliah E, Xia Y, Iwai A, Yoshimoto M, Otero DA, Kondo J, Ihara Y and Saitoh T (1993) Molecular cloning of cDNA encoding an unrecognized component of amyloid in Alzheimer disease. *Proc Natl Acad Sci U S A* 90:11282-11286.

Uversky (2007) Neuropathology, biochemistry, and biophysics of alpha-synuclein aggregation. *J Neurochem* 103:17-37.

van Marle G, Henry S, Todoruk T, Sullivan A, Silva C, Rourke SB, Holden J, McArthur JC, Gill MJ and Power C (2004) Human immunodeficiency virus type 1 Nef protein mediates neural cell death: a neurotoxic role for IP-10. *Virology* 329:302-318.

Waak J, Weber SS, Waldenmaier A, Gorner K, Alunni-Fabbroni M, Schell H, Vogt-Weisenhorn D, Pham TT, Reumers V, Baekelandt V, Wurst W and Kahle PJ (2009) Regulation of astrocyte inflammatory responses by the Parkinson's disease-associated gene DJ-1. *FASEB J* 23:2478-2489.

Wahner AD, Sinsheimer JS, Bronstein JM and Ritz B (2007) Inflammatory cytokine gene polymorphisms and increased risk of Parkinson disease. *Arch Neurol* 64:836-840.

Wallace DR, Dodson SL, Nath A and Booze RM (2006) Delta opioid agonists attenuate TAT(1-72)-induced oxidative stress in SK-N-SH cells. *Neurotoxicology* 27:101-107.

Wang CN, Shiao YJ, Lin YL and Chen CF (1999) Nepalolide A inhibits the expression of inducible nitric oxide synthase by modulating the degradation of IkappaB-alpha and IkappaB-beta in C6 glioma cells and rat primary astrocytes. *Br J Pharmacol* 128:345-356.

Weiss T, Grell M, Siemienski K, Muhlenbeck F, Durkop H, Pfizenmaier K, Scheurich P and Wajant H (1998) TNFR80-dependent enhancement of TNFR60-induced cell

death is mediated by TNFR-associated factor 2 and is specific for TNFR60. *J Immunol* 161:3136-3142.

Whiteside ST and Israel A (1997) I kappa B proteins: structure, function and regulation. *Semin Cancer Biol* 8:75-82.

Whitton PS (2007) Inflammation as a causative factor in the aetiology of Parkinson's disease. *Br J Pharmacol* 150:963-976.

Williams R, Dhillon NK, Hegde ST, Yao H, Peng F, Callen S, Chebloune Y, Davis RL and Buch SJ (2009a) Proinflammatory cytokines and HIV-1 synergistically enhance CXCL10 expression in human astrocytes. *Glia* 57:734-743.

Williams R, Yao H, Dhillon NK and Buch SJ (2009b) HIV-1 Tat co-operates with IFN-gamma and TNF-alpha to increase CXCL10 in human astrocytes. *PLoS One* 4:e5709.

Wilms H, Rosenstiel P, Sievers J, Deuschl G, Zecca L and Lucius R (2003) Activation of microglia by human neuromelanin is NF-kappaB dependent and involves p38 mitogen-activated protein kinase: implications for Parkinson's disease. *FASEB J* 17:500-502.

Wilms H, Rosenstiel P, Romero-Ramos M, Arlt A, Schafer H, Seegert D, Kahle PJ, Odoy S, Claasen JH, Holzknecht C, Brandenburg LO, Deuschl G, Schreiber S, Kirik D and Lucius R (2009) Suppression of MAP kinases inhibits microglial activation and attenuates neuronal cell death induced by alpha-synuclein protofibrils. *Int J Immunopathol Pharmacol* 22:897-909.

Wyss-Coray T and Mucke L (2002) Inflammation in neurodegenerative disease--a double-edged sword. *Neuron* 35:419-432.

Xia MQ, Bacskai BJ, Knowles RB, Qin SX and Hyman BT (2000) Expression of the chemokine receptor CXCR3 on neurons and the elevated expression of its ligand IP-10 in reactive astrocytes: in vitro ERK1/2 activation and role in Alzheimer's disease. *J Neuroimmunol* 108:227-235.

Xiao G, Harhaj EW and Sun SC (2001) NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100. *Mol Cell* 7:401-409.

Xu J, Kao SY, Lee FJ, Song W, Jin LW and Yankner BA (2002) Dopamine-dependent neurotoxicity of alpha-synuclein: a mechanism for selective neurodegeneration in Parkinson disease. *Nat Med* 8:600-606.

Yang GY, Zhao YJ, Davidson BL and Betz AL (1997) Overexpression of interleukin-1 receptor antagonist in the mouse brain reduces ischemic brain injury. *Brain Res* 751:181-188.

Yao Y, Kubota T, Sato K, Takeuchi H, Kitai R and Matsukawa S (2005) Interferons upregulate thymidine phosphorylase expression via JAK-STAT-dependent transcriptional activation and mRNA stabilization in human glioblastoma cells. *J Neurooncol* 72:217-223.

Yasuda Y, Shimoda T, Uno K, Tateishi N, Furuya S, Yagi K, Suzuki K and Fujita S (2008) The effects of MPTP on the activation of microglia/astrocytes and cytokine/chemokine levels in different mice strains. *J Neuroimmunol* 204:43-51.

Yeruva S, Ramadori G and Raddatz D (2008) NF-kappaB-dependent synergistic regulation of CXCL10 gene expression by IL-1beta and IFN-gamma in human intestinal epithelial cell lines. *Int J Colorectal Dis* 23:305-317.

Yin L, Ohtaki H, Nakamachi T, Kudo Y, Makino R and Shioda S (2004) Delayed expressed TNFR1 co-localize with ICAM-1 in astrocyte in mice brain after transient focal ischemia. *Neurosci Lett* 370:30-35.

Yuan Y, Jin J, Yang B, Zhang W, Hu J, Zhang Y and Chen NH (2008) Overexpressed alpha-synuclein regulated the nuclear factor-kappaB signal pathway. *Cell Mol Neurobiol* 28:21-33.

Zaheer A, Zhong W, Uc EY, Moser DR and Lim R (1995) Expression of mRNAs of multiple growth factors and receptors by astrocytes and glioma cells: detection with reverse transcription-polymerase chain reaction. *Cell Mol Neurobiol* 15:221-237.

Zareba M, Bober A, Korytowski W, Zecca L and Sarna T (1995) The effect of a synthetic neuromelanin on yield of free hydroxyl radicals generated in model systems. *Biochim Biophys Acta* 1271:343-348.

Zecca L, Costi P, Mecacci C, Ito S, Terreni M and Sonnino S (2000) Interaction of human substantia nigra neuromelanin with lipids and peptides. *J Neurochem* 74:1758-1765.

Zecca L, Zucca FA, Wilms H and Sulzer D (2003) Neuromelanin of the substantia nigra: a neuronal black hole with protective and toxic characteristics. *Trends Neurosci* 26:578-580.

Zecca L, Zucca FA, Albertini A, Rizzio E and Fariello RG (2006) A proposed dual role of neuromelanin in the pathogenesis of Parkinson's disease. *Neurology* 67:S8-11.

Zecca L, Casella L, Albertini A, Bellei C, Zucca FA, Engelen M, Zadlo A, Szewczyk G, Zareba M and Sarna T (2008a) Neuromelanin can protect against iron-mediated oxidative damage in system modeling iron overload of brain aging and Parkinson's disease. *J Neurochem* 106:1866-1875.

Zecca L, Wilms H, Geick S, Claasen JH, Brandenburg LO, Holzknecht C, Panizza ML, Zucca FA, Deuschl G, Sievers J and Lucius R (2008b) Human neuromelanin induces neuroinflammation and neurodegeneration in the rat substantia nigra: implications for Parkinson's disease. *Acta Neuropathol* 116:47-55.

Zhang W, Wang T, Pei Z, Miller DS, Wu X, Block ML, Wilson B, Zhang W, Zhou Y, Hong JS and Zhang J (2005) Aggregated alpha-synuclein activates microglia: a process leading to disease progression in Parkinson's disease. *FASEB J* 19:533-542.

Zhang W, Phillips K, Wielgus AR, Liu J, Albertini A, Zucca FA, Faust R, Qian SY, Miller DS, Chignell CF, Wilson B, Jackson-Lewis V, Przedborski S, Joset D, Loike J, Hong JS, Sulzer D and Zecca L (2011) Neuromelanin activates microglia and induces degeneration of dopaminergic neurons: implications for progression of Parkinson's disease. *Neurotox Res* 19:63-72.

Zhao ML, Kim MO, Morgello S and Lee SC (2001) Expression of inducible nitric oxide synthase, interleukin-1 and caspase-1 in HIV-1 encephalitis. *J Neuroimmunol* 115:182-191.

Zhou W, Schaack J, Zawada WM and Freed CR (2002) Overexpression of human alpha-synuclein causes dopamine neuron death in primary human mesencephalic culture. *Brain Res* 926:42-50.

Zlotnik A and Yoshie O (2000) Chemokines: a new classification system and their role in immunity. *Immunity* 12:121-127.

Zucca FA, Giaveri G, Gallorini M, Albertini A, Toscani M, Pezzoli G, Lucius R, Wilms H, Sulzer D, Ito S, Wakamatsu K and Zecca L (2004) The neuromelanin of human substantia nigra: physiological and pathogenic aspects. *Pigment Cell Res* 17:610-617.

VITA

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**Major Field**: Biomedical Sciences

**Scope and Method of Study**:

The scope of this dissertation was to study the effects of two Parkinson's disease associated molecules, neuromelanin and α-synuclein, on inflammatory signaling and expression in A172 astroglial cells in vitro. Real-time PCR and ELISA were used to measure changes in CXCL10 mRNA and protein expression, respectively, and the Griess reagent was used to measure nitrite accumulation as an index of iNOS expression. NF-κB activation was examined using ELISA-based transcription factor assays.

**Findings and Conclusions**:

The findings from the current study concluded a cytokine-dependent regulation of astroglial CXCL10 and iNOS expression by α-synuclein and neuromelanin. Alpha-synuclein induced the expression of CXCL10 in both IL-1β- and TNFα-stimulated astroglial cells, however, this enhancement by α-synuclein was not demonstrated for the mRNA. Studies revealed the ability of α-synuclein to mediate an increase in mRNA stability, which may be, in part, the mechanism responsible for the increase in CXCL10 protein expression. The NF-κB signaling pathway was not activated by α-synuclein under similar treatments that induced CXCL10 expression yet was activated in the presence of other α-synuclein concentrations, suggesting the expression of other inflammatory mediators besides CXCL10 may be modulated by α-synuclein. Alpha-synuclein also increased astroglial iNOS expression, further supporting the inflammation-mediated effects by α-synuclein.

Investigation of role of NM on astroglial chemokine expression revealed a down-regulation of CXCL10 protein expression with a reduction in NF-κB activation, suggesting a possible mechanism responsible for decrease in CXCL10 protein. NM enhanced cytokine-induced iNOS expression in the presence of astrocytes; however, further studies need to be conducted to understand the mechanism.

Overall, the findings provide an initial understanding of the effects of PD-associated molecules and inflammation on human brain cells. The regulation of astroglial-derived inflammatory mediators by extracellular α-synuclein and neuromelanin may play a role in PD-associated neuroinflammation. The observations provide novel information that may be useful in identifying molecular targets for therapeutic intervention in PD.

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